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CONTRIBUTIONS

FROM THE

UNIVERSITY LABORATORY FOR MEDICAL BACTERIOLOGY

TO CELEBRATE THE INAUGURATION OF THE

STATE SERUM INSTITUTE

EDITED BY

CARL JUL. SALOMONSEN

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STATENS SERUM-INSTITUT er en Aflægger af UNIVERSITETETS LABORATORIUM FOR MEDICINSK BAKTERIOLOGI.

Gjennem dette Festskrift sender Moder-Laboratoriet sin Hilsen til det nye Institut med de bedste Ønsker for dets fremtidige Virksomhed i Videnskabens og Lægekunstens Tjeneste.

København, September 1902.

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I.

CARL JUL. SALOMONSEN

THE RISE AND GROWTH OF THE STATE SERUM INSTITUTE

IN the autumn of 1894, after Roux's publication of the first decisive experiments with Behring's serotherapy applied to diphtheria, the production of antidiphtheric serum was eagerly taken in hand everywhere. In Denmark, too, the first introductory steps were made, at that time, towards the foundation of the Serum Institute, which to-day is an accomplished fact. As the director of the University Laboratory of Medical Bacteriology I felt it my duty to study more thoroughly the technic of the production of antidiphtheric serum. So, in September 1894, I went to the Pasteur Institute, the only laboratory where for years they had both been working at the manufacture of serum and allowed other people to share the experience they had gained. Immediately after my return, I sent in a petition to the Ministry of Ecclesiastical and Educational Affairs, asking that an extra grant of kr. 10 000 should be made in the budget of 1895—96, to enable the Laboratory to make serotherapeutic experiments, while it, at the same time, undertook

1st to have in view, as far as possible, during the experiment, the production of great quantities of anti-diphtheric serum;

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2dly, to take care that some young medical men should be thoroughly trained in the technic which is, on the whole, common to the manufacture of the different anti-sera;

3dly, to dispense, without payment, to such hospitals and doctors as might require it, all the serum antidiphthericum, which, under the circumstances, the laboratory was able to produce.

The grant asked for being given, I thought it most proper to use part of it for *establishing a special sero-therapeutic department to be independent of the students' laboratory* and to have, to a certain extent, an equipment and a staff of its own. It should then be left with the future fate of serum-therapy to decide *whether* this new department should be relinquished, its premises and apparatus being incorporated with the students' laboratory as a useful extension of the same — *or* whether it should be necessary to extend it further, separate it from the students' laboratory and thus make it into an independent institution.

The Laboratory could only place two very small rooms and a tolerably good guinea-pig house at the disposal of the new Department. Before long, however, it became necessary to find room for small animals outside the premises, at the Zoological Garden, which is rather far away, where a special little breeding-house was erected for the guinea-pigs. The horses, too, had to be stabled far away from the Laboratory, in the stables of the Veterinary College, kindly put at our disposal. Furthermore, for a short time, the Department has had the use of a stable with ten stalls, belonging to a tram-car company, for some experiments on a somewhat extensive scale. A few years ago a small out-of-the-way room at the Laboratory was equipped for plague experiments, and added to the Department. As a matter of course the other rooms of the Laboratory have always been at the disposal of the Department to be used for scientific researches; but it is evident, from what has been said above, the conditions, under which work has been carried on, have been anything but favourable.

As members of the staff an assistant and a helper without any medical training were appointed; later on a consulting veterinary was added. As an assistant, Dr. Th. Madsen, was appointed. He has during the last years conducted the work of the Department, and will be appointed chief of the Serum-Institute laboratory.

The work in this small sero-therapeutic laboratory took a somewhat different turn from what was expected at the outset. Indeed the difficulties and uncertainties of the serum production soon proved much greater than we supposed would be the case. It must be remembered that at that time the few existing serum manufactories which possessed a greater experience in the matter than ourselves, had, in the interest of their business, preserved an absolute silence with regard to their methods and results. Thereby the difficulties, connected with the work in the Sero-therapeutic Department, were greatly increased. We had here to pass through the same painful experiences with regard to the production of strong toxin and strong serum as they had elsewhere. The race of diphtheria bacillus that one month will make a toxin of great strength, may by the next have lost its toxigenic power, and as to the strength of the serum, that is evidently dependent, not only upon the individuality of the horse, „strength“ of the toxin, mode of immunising and time of bleeding but upon other

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circumstances as well, of which we possess as little knowledge as of the varying toxigenic power of the diphtheria bacillus. It is this enigmatical uncertainty on all the chief points, that makes the production of serum both very expensive and very precarious. The Laboratory has during the past years left no stone unturned to gain light on these points. But it has met with no greater success in arriving at fully satisfactory methods of work than the many other European or American serum laboratories which have, of recent years, placed their experience at our disposal. For that reason I shall refrain from entering into particulars with regard to the technique used in the Deparment. The more so as, in working it out, we have had to consider the very unfavourable conditions under which the Department had to work. I will only state that our method of immunisation follows very closely upon that applied by Dr. Dean at the Jenner Institute for Preventive Medicine. It is Dr. Dean, too, whom we have to thank for so kindly supplying us with the race of diphtheria bacillus (Park & Williams no. 8) which we are now using. The methods of measuring are, of course, those of Ehrlich. This eminent pathologist has, during the whole existence of the Department, assisted us in every possible way, and I take this opportunity to express to him my deepfelt gratitude.

Even though our Laboratory is as ignorant of the conditions under which strong toxin and strong antidiphtheric serum are produced as all other laboratories, we have, no less than they, succeeded, during our continuous work, in increasing the strength of the serum to a much higher degree than formerly.

In June 1895, the first antidiphtheric serum produced in the Department was sent to the *Blegdam Hospital* of Copenhagen. Soon after, the Laboratory began to, fulfil the obligation which it had accepted, viz. to *dispense antidiphtheric serum free of charge*, as far as its stock allowed.

Unfortunately it was soon seen that the above-named difficulties made it impossible to meet all demands. As early as in November 1895, the dispensation of serum had to cease for a time, in order to allow the Laboratory to increase the strength of the serum and to immunise a large number of horses. Since then it has certainly not been necessary to cease the dispensation of serum, but during the first few years the Laboratory has had to exert itself to the utmost in order to satisfy the numerous demands made from all parts of the country. In later years the productiveness of the Department has been steadily increasing. The following table shows the quantity of serum which has been dispensed gratis by the Department during the last two years:

	flasks containing	Imm. Units.
From the 1st July 1900 till the 1st July 1901	5 036	17 153 000
From the 1st July 1901 till the 1st July 1902	6 305	24 133 000

In 1896 the Director of the Laboratory sent out a printed form of questions to all the doctors who had been supplied with serum, in order to obtain their opinion of the new remedy. The answers received showed that, as a rule, the results of the serum treatment in Denmark gave general satisfaction. The reports sent in to the Board of Health from the medical officers of health were to the same effect. We have also a striking proof from the well-known series of experiments undertaken on a large scale by Professor J. Fibiger at

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the Blegdam Hospital with our serum, and which showed such unmistakable results.

Antidiphtheric Serum is the only serum which has been produced in the Department on a large scale for therapeutic purposes. For the rest, the Department has not restricted itself to the manufacture of serum. It has tried, in different ways, to be of use to „the Authorities of Health“, for instance, by undertaking the bacteriological diagnosis of doubtful cases af plague in Denmark, and it has further been of assistance in different ways to the University in its teaching of bacteriology. It has not itself undertaken any teaching, apart from the fact that some foreign colleagues have, under Dr. Madsen:s guidance, made themselves acquainted with the technique of the manufacture of serum or a part of the same.

Scientific work has also been constantly carried on by the Department, as may be seen from the following list of publications issued during the last years.

MADSEN (Th.). Experimentelle Undersøgelser over Difterigiften. Diss. Kbhv. 1896. — Also publ. in Zeitsch. f. Hygiene. Bd. 24 og 25. 1897.

SALOMONSEN (C. J.) and MADSEN (TH.). Recherches sur la marche de l'immunisation active contre la diphtérie. (Bull. de l'Ac. R. des Sc. et des Lettres de Danemark 1896. — Also publ. in Ann. de l'Inst. Pasteur 1897. Vol. XI.).

SALOMONSEN (C. J.) and MADSEN (TH.). Om Forskjelligheder i Serums antidifteriske Styrke hos aktivt immuniserede Heste. [On differences in the strength of antidiphtheric serum in actively immunised horses.]. (Nordiskt medicinskt Arkiv. 1897. Festbind for Axel Key.).

SALOMONSEN (C. J.) and MADSEN (TH.). Om individuel Prædisposition til Antitoxindannelse. [On individual predisposition to produce antitoxin]. (Bull de l'Ac. R. des Sc. et des Lettres de Danemark. 1898).

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SALOMONSEN (C. J) and MADSEN (TH.). Om nogle Giftes Indvirkning på Blodets antitoxiske Styrke. (Ibidem 1898. — Also publ. in C. r. de l'Ac. des Sc. Paris. 1898).

SALOMONSEN (C. J.) and MADSEN (TH.). Om Regeneration af Antitoxin efter store Blodudtømmelser. (Ibidem 1898. Also publ. in Ann. de l'Inst. Pasteur 1898).

SALOMONSEN (C. J.) and MADSEN (TH.). Recherches sur la marche de l'immunisation active contre la diphtérie. 2^me^ mémoire. (Ann. de l'Inst. Pasteur 1899. Vol. XIII).

MADSEN (TH.). Om Difterigiftens Konstitution. [On the constitution of diphtheria toxin]. (Bull. de l'Ac. R. des Sc. et des Lettres de Danemark 1899, also publ. in Ann. de l'Inst. Pasteur. 1899).

MADSEN (TH.). Om Toxiner og Toxinmodificationer. [On toxins and their modifications] (Hospitalstidende. 1899).

SALOMONSEN (C. J.). Fremstilling og Uddeling af antidifterisk Serum i Danmark I—II. 1897—99. [Fabrication and dispensation of antidiphtheric serum in Denmark].

DREYER (GEORGES). Experimentelle Undersøgelser over Difterigiftens Toxoner. Diss. Kbhv. 1900. [Experimental researches on diphtheria toxons].

DREYER (GEORGES). Recherches sur la fixation dans l'organisme de la toxone diphtérique. (Bull. de l'Ac. R. des Sc. et des Lettres de Danemark. 1900).

MADSEN (TH.). Sur les toxones (XIII Congrés international de médecine. Paris 1900. Section de Bactériologie et de Parasitologie).

MADSEN (TH.) and DREYER (GEORGES). Sur l'immunisation à l'aide des toxones. (Bull. de l'Ac. R. des Sc. et des Lettres de Danemark 1901, cfr. XIII Congrès international de médecine, Paris 1900. Section de Bactériologie et de Parasitologie: DREYER. Sur l'immunisation à l'aide des toxones).

MADSEN (TH.). Antitoxins and Agglutinins. Some analogous phenomena of the variation of production arising during active and passive immunisation. Communicated at the Pan-American Medical Congress. Buffalo. Septb. 1901.

ARRHENIUS (SVANTE) and MADSEN (TH.). Physical chemistry applied to the study of toxins and antitoxins. 1902.

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ARRHENIUS (SVANTE) and MADSEN (TH.). On the molecular weight of diphtheria toxin. 1902. *

DREYER (GEORGES) and MADSEN (TH.). Studies on diphtheria toxin. 1902. *

MADSEN (TH.) and JØRGENSEN (AXEL). Typhoid and cholera agglutinins. Their fate during active and passive immunisation.

LEVIN (ERNST). Coli agglutinins. The course of their formation. 1902. *

MADSEN (TH.). A formula indicating the mode of decrease of antibodies in the organism. *

DREYER (GEORGES). A new apparatus for rapid delivery of a given quantity of fluid. *

(The papers marked with asterisks are published in the present volume.)

As already stated, the Sero-therapeutical Department soon proved too restricted to satisfy all the great demands made upon it. This was felt on all sides, and if the work has been kept going, up till now, with comparatively good results, under such conditions, it is due partly to the readiness with which the Veterinary School has supplied stabling, partly to the circumstance that one can work under very bad conditions, when there is a chance of their soon mending.

In the spring of 1898 there was such a chance, when a large number of leading doctors in the hospitals, at the suggestion of Dr. O. Bruun of Esbjerg, sent in a request asking me to take steps to enable the Laboratory to distribute, regularly and uninterruptedly, all the serum antidiphthericum required in this country. Of course I at once sent on the request to the Ministry of Education, asking them to take the matter in hand. The question soon arose as to whether the *Sero-therapeutic Department* should be extended while it remained attached to the University Laboratory or whether it should be *transformed into an independent institution* which, at the same time, was to be removed from the University and placed under the Ministry of Justice which is responsible for public health in this country.

In June 1898 the Medical Faculty pronounced in favour of the second alternative in a memorandum, from which I am going to quote the following extracts, as they clearly show the points of view from which the State Serum Institute was founded and arranged.

> For several reasons the Faculty considers it most desirable, that *the State should continue manufacturing the antidiphtheric serum,* carrying on the work that has been done in the Sero-therapeutic Department and, if necessary, extending it so as to enable it to produce the large quantities of serum that the leading doctors of the hospitals are so eagerly asking for in the interest of their patients . . . .
>
> The manufacture of serum can only be carried on in an Institute which is provided with an *experimental pathological laboratory,* and it will naturally be the object of the Director of the Institute not only to supply the necessary antidiphtheric serum to the State, but also to keep in touch with and work at all subjects of a kindred nature, in short, attend to all investigations *which bear upon the use of bacterial poisons and antidotes in the service of hygiene and therapeutics.* Now, experience shows that these investigations (the importance of which for practical medicine, too, is increasing day by day) can only be made in institutions working *with an extensive material* and relatively great funds. To give up the production in this country really means the same as to entirely exclude our medical profession from taking part in this most important work.
>
> Whereas the Faculty must strongly advise that the State should go on manufacturing antidiphtheric serum to the extent which is wanted to cover the consumption of the country, thus taking care that the production of serum takes place in a fully equipped experimental pathological institution the Faculty also *shares the scruples* of the Ministry, as expressed in its letter of the

9th inst., *as to handing over this task to the University Laboratory for Medical Bacteriology.* It would be possible, of course, to so extend the Sero-therapeutic Department as to enable it to supply the whole country with antidiphtheric serum . . . . The Faculty, however, as already stated, has scruples about making a proposal to that effect, as such a departement, even though it might be of great importance as an institution for investigation and instruction, would have, as its chief object, one with which the authorities responsible for public health, are concerned; but especially as it is impossible, for the present, to tell how extensive the work of such a serum institution would be in the presence of the rapid development of experimental pathology.

The Faculty therefore most earnestly begs to recommend that steps should be taken *to found a fully equipped Serum Institute under the jurisdiction of the Ministry of Justice.*

The future position of the Sero-therapeutic Department would then naturally depend on the position and organisation of the Serum Institute. In fact, for the last four years, the Sero-therapeutic Department has proved to be of very great importance, from an educational point of view and to the work generally of the University Laboratory for Medical Bacteriology, through the rich material such a department always offers. It would therefore greatly impair the work of the Laboratory as a training institution, if the department was relinquished, *unless, at the same time, the Laboratory was allowed the use of the material of the Serum Institute* for teaching purposes. It would hardly be a difficult thing to find *an arrangement that would make it possible for the two institutions to work together,* although under the jurisdictions of two different Ministries. If such a co-operation was brought about the Sero-therapeutic Department would, of course, have to be relinquished.

Both the Ministry of Education and the Ministry of Justice being in agreement with this view, the Director of the Laboratory was, in October 1898, asked by the latter to make out a plan for a State institution for production of antidiphtheric serum.

Being of opinion that it would greatly advance matters if, at the time of sending in the plan to the Ministry of Justice, a suitable site was also offered to the Ministry, I had already, early in the summer, addressed a petition to the Ministry of War, which have at its disposal extensive plots of land in the neighbourhood of the Laboratory, asking it to present the University Laboratory for Medical Bacteriology with a building plot not smaller than 10 000 □ ells, for the erection of a Serum Institute. General Tuxen, then Ministry of War, received this request in a very kind way. In October 1898 the Laboratory was promised a piece of land of the size stated, and in November 1898 the proposal which led to the erection of the Institute as it stands now, was sent to the Ministry of Justice.

Unfortunately a Bill on the matter was not brought in that year, but was deferred to the next parliamentary session when, however, for political reasons, it did not make progress.

In the following session 1900—1901 the Bill was brought in again, and passed in the spring 1901; the royal assent to the Act was given on the 20th of March.

The Act is worded as follows:

STATE SERUM INSTITUTE ACT.

Art. 1.

An Institute for the production of antidiphtheric serum shall be established under the jurisdiction of the Ministry of Justice. It shall undertake to supply such serum on the largest possible scale to the medical men of this country and its colonies, on appliance for it to the said Institute.

Art. 2.

A sum of kr. 172 800 shall be used for the erection of the necessary buildings on a site of 10 000 □ ells, situated to the north of the barracks belonging to the 2nd regiment of artillery, on the Common of Amager, as well as for the equipment of the said Institute.

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Art. 3.

The staff of the said Institute, to be appointed when the work commences, shall consist of the following: 1st, a Director appointed by the Ministry of Justice. He shall have the chief management of the said Institute with a yearly salary of 1000 kroner; 2dly, a Chief of the laboratory to be appointed by the King. He shall conduct the manufacture of the serum and undertake the work connected with it as well as all scientific work and investigations that may occur. He shall have his residence at the said Institute and a yearly salary of 3200 kr. to be increased at the rate of 600 kroner every five years, such salary, however, not to exceed 5600 kroner.

A sum of 3960 kroner a year may be set apart for assistants to be engaged by the said Director.

All other expenses to be used for the working of the said Institute shall be decided by the yearly budget.

Art. 4.

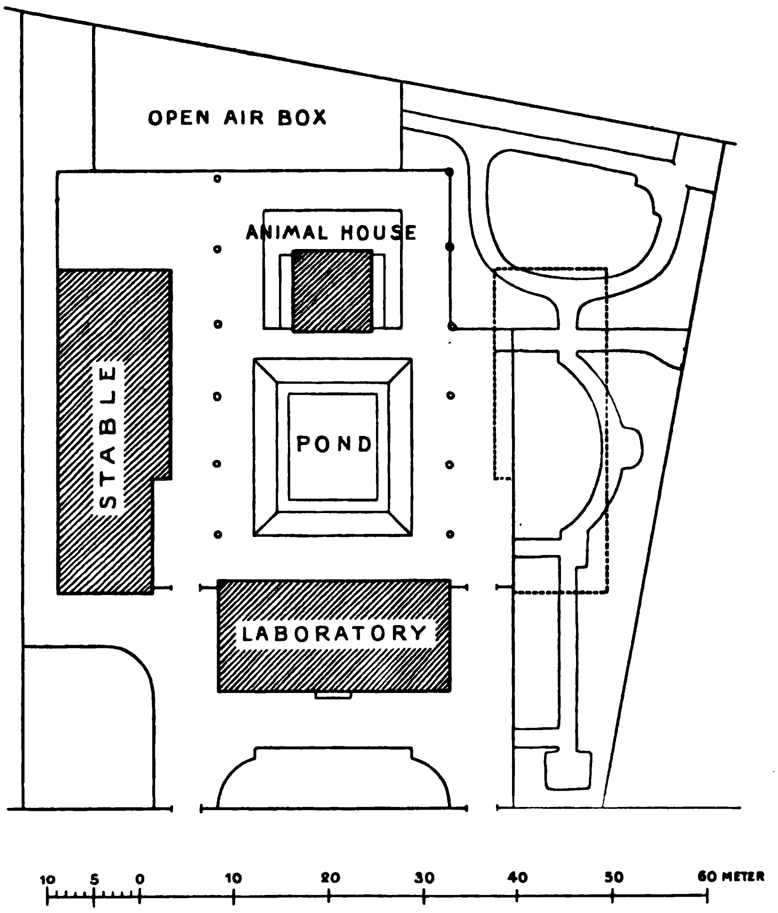
The Ministry of Justice shall be authorized to fix, for periods not exceeding five years, the price at which antidiphtheric serum shall be sold by the said Institute.

Art. 5.

The Ministry of Justice shall be authorized to fix the date when the said Institute shall begin its work, and to spend a sum not exceeding 15 000 kroner on preparatory work, especially to be used for the purchase of the necessary quantity of suitable horses.

In one point this law differs from the Bill as proposed by the Government. According to the Bill antidiphtheric serum was to be supplied *gratis* as before to everyone, whereas the Act says that serum shall be *sold* by the Laboratory. The Ministry of Justice, however, with the consent of the newly elected Rigsdag, has put the price so low, that there is practically no rupture with the old system.

The site given by the Ministry of War is about 14 000 □ ells. It is situated in the neighbourhood of the University Laboratory for Medical Bacteriology on the small island of Amager, just outside the old fortifications of the city. The northern limit of the site is laid out in accordance with the building-plan prepared by the Copenhagen Town Council for the surrounding ground, so that the Institute, when the plan is entirely carried out, will be situated on a large boulevard. As will be seen from the annexed plan of the site, there will be a main building, a stable and a house for small animals.



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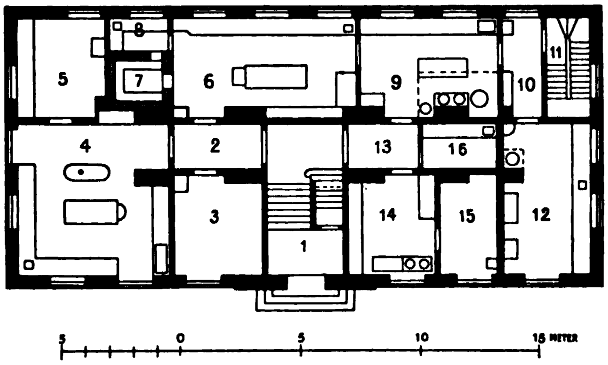
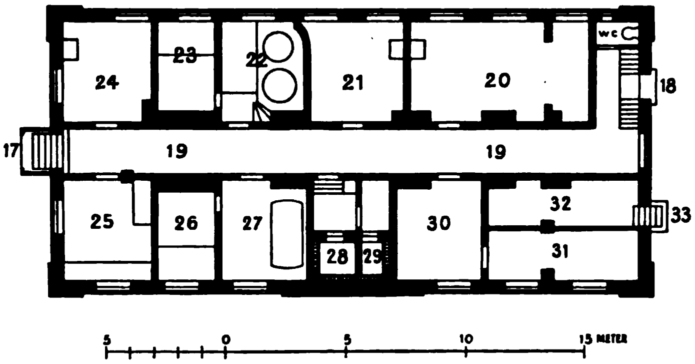
The main-building is composed of basement, ground-floor, first floor and garret. The laboratory occupies the basement, the ground-floor and part of the garret. The first floor is reserved as a private residence for the Chief of the laboratory.

The two plans annexed will show the size and arrangement of the premises. From the main staircase (1) you pass through the corridor (2) into the *Chemical Laboratory* (4) adjoining the *Weighing-room* (5). From this corridor there is another entrance into the *Bacteriological Laboratory* (6). In the wall of the latter, on the east side, is the entrance into the *Incubator-room* (7); another door leads into the small *Sowing-room* (8) in which the flasks are inoculated and, by means of a trapdoor, passed directly into the Incubator-room. From the Bacteriological Laboratory you pass into the room (9) where the media are prepared, and where the smaller and larger autoclaves and other sterilising apparatus are placed. Through an anteroom (10), to which there is also an entrance from the back staircase (11), you reach the so-called *Research Laboratory* (12), which is designed for work with such infectants as must be carefully kept away from the rooms in which antidiphtheric



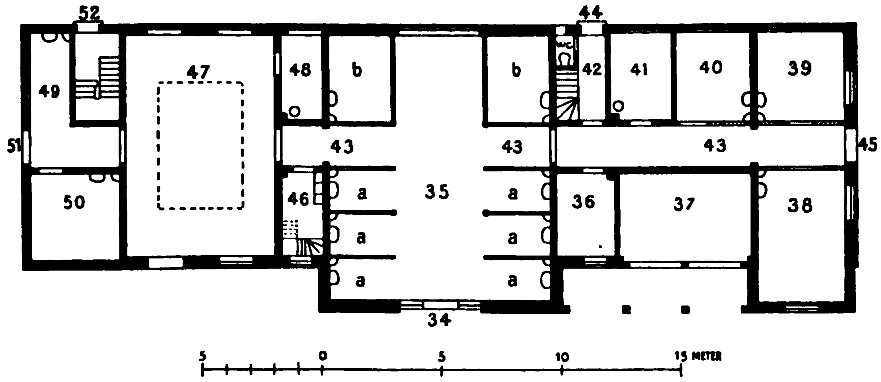
serum is made, the only admittance being through the anteroom (10); it is entirely isolated from the other parts of the building.

When entering from the main staircase through the corridor (13), one reaches the two rooms 14 and 15. These are to be used for treating the blood which is obtained from the bloodletting, and which, after coagulation and separation of the serum, may be brought directly into room 14 to be egouttated and filtered.



The Serum is bottled in room 15, which is exclusively used for that purpose. Adjoining the corridor (13) there is also a small *dark room* (16), set apart for researches and preparations requiring the exclusion of daylight. On the ground-floor, next to the main staircase, is the *Director's room* (3), with an entrance from the corridor (2).

On the east and the west sides there are special ways down (17) (18) into the *basement* besides those leading from the main and the back staircases. The way down (17), on the east side, takes you directly into the broad corridor (19), which divides the basement into two equal parts. On the south side there are two spacious rooms (20) (21) for *small animals*, and a third room (24) to be used for *isolating* such animals, if desired. Room 22 contains the boilers of the *central heating apparatus.* On the north side of the corridor are the *washing up room* (25), *workshop* (26), *wood cellar* (27) and the *electric plant* supplying the whole building with light and motor power. In room 31 a gas engine and a dynamo are placed. The electric accumulator is placed in a small room (32) completely separated from the rest of the basement; it can only be reached by the small staircase (33).



On each side of the corridor there is a *heating room* (23) (26), and under the staircase a *cooling room,* divided into two parts (28) (29), both of which are to be principally used for the storing of toxin and serum.

One of the attics is to be used as a common room for the subordinates of the staff, not living at the Institute, the others for a laundry, for stores, etc.

The *stables* are situated in the immediate neighbourhood of the main building. A doorway (34) in the west wall leads into the *main stable* (35) containing six stalls (a) and two loose boxes (b); from a small adjoining *fodder room* (46) a flight of stairs leads to the *hay-loft* above. In the south wing of the building there are three roomy boxes (38) (39) (40), two of which (39) (40) are intended for goats and are connected with an enclosure in the open-air. Here are also further to be found a *harness-room* (36), a *coach-house* (37), a small room (41) for researches best undertaken in the stable itself, in the immediate proximity of the animals, and a small passage (42) from which there is a way out into the open air and a staircase leading to the apartments occupied by the stable man and the caretaker. From the *central passage* (43) there is, on the south side, an exit (45) into the open air; and at the other end a door opening into the *bleeding room* (47). This occupies the height of the two stories of the building and receives light from above. In the adjoining room (48) the drawn blood is left for coagulation to take place and for the subsequent preparatory treatment. Apart from the rest of the stables is *a small stable* with one stall (48) and a loose box (49) for sick horses; it has a special entrance (50) on the north side and is to be used *for isolating* suspected cases amongst the serum horses. Above the isolating stable there are a few rooms for an *assistant*, and a bathroom for the subordinates of the staff; all these rooms are reached through the door (51) on the east side.

The *house* intended *for small animals* (rabbits, guinea-pigs, rats, mice, poultry, pigeons, etc.) contains a single room with a terrazzo floor on which the guinea-pigs can be kept in movable wooden frames. The animal house is enclosed on three sides by a yard paved with concrete. The animals pass into this through a number of small openings in the two side-walls. On each of these walls, at some distance from the ground, a number of *rabbit hutches* are arranged. In the middle of the ground (see plan of site) there is a *pond* for aquatic birds, frogs, and other animals.

On the part of the ground not occupied by buildings

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is an *enclosure* for horses (see plan of site). The remainder of the ground, sufficient in extent to allow another large stable being built some future day (see plan of site), is provisionally laid out as a *garden*.

The above-named enclosure being rather small, the Institute has hired and enclosed an adjoining piece of land of the size of 33 □ meters where there is plenty of room for exercising the horses.

For the working of the Institute (comp. the Act, art. 3, last paragraph) provisionally a yearly sum of 23000 Kr. has been voted.

The Institute is now sufficiently large and well equipped to meet all the requirements of the present time, and if, at some future date, theoretical and applied Bacteriology should make a fresh start and develop at the same rate as it has done during the last twenty five years of the nineteenth century, there will be, I trust, ample room for all necessary additions on the site of the Institute.

II.

CARL·JUL. SALOMONSEN

GENERAL PATHOLOGY AS A UNIVERSITY SUBJECT

One of the chief aims of the Medical Bacteriological Laboratory from the first day of its establishment — now nearly twenty years ago — has been to impress upon the University of Copenhagen the importance of general pathology as a special branch of study. It has tried its best to raise the necessary funds and to start teaching within the limits of its means and appliances. This is, by no means, the first time that I have publicly expressed my opinion with regard to this matter. So I may perhaps be allowed, by way of an introduction, to quote from the „Tidskrift for praktisk Medicin"[1]) the concluding part of an address I delivered at a medical congress held in Copenhagen in 1888.

> „General pathology! This Cinderella among university subjects that, at most European universities, has no dwelling of her own, being obliged to seek shelter now at the house of therapeutics, now with pathological anatomy, now in the splendid palaces of hygiene, general pathology who has known better days! There was a time when it filled the post of honour, when people were foolish enough to be content with driving its principles into the students — clinical instruction being almost nil. Justice was done to clinical subjects at last; they obtained the position they ought always to have occupied. For several years they have, no doubt greatly to the benefit of

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the patients, been split up into no end of specialities. But, at the same time, general pathology as a university subject has been sinking into a more and more subordinate position, from which not even the genius of Cohnheim has been able to raise it. This state of things cannot last for ever. When, at a future date, classical surgery has been broken up into its component parts, ophthalmology, gynecology, otology, orthopedy, laryngology etc.; when the physicians have distributed among themselves the viscera, handing over the lungs to one man, the kidneys to another, the heart to this, the brain to that man; when the whole of this natural development has been completed and has found a permanent expression in the order in which the subjects are arranged for teaching purposes, then, no doubt, a want will be felt for a subject which, like an *Alma Mater*, will be able to gather her scattered brood under her wings. It is the subject of General Pathology alone that can and will supply this need.“

During the intervening fourteen years, a great deal has been gained, but much still remains to be done.

A great deal has been gained: A chair of general pathology has been founded to rank among the ordinary professorships of the University. Attached to this post is a tolerably good bacteriological laboratory with two assistants and accommodation for thirty-three students. A medico-bacteriological course is now part of the medical curriculum. For the last seven years a Serotherapeutic Department has been attached to the Laboratory, allowing researches on immunity to be made on a large scale, and this Department, I hoped, would develop into one for applied medical bacteriology also serving for purposes of teaching. Finally by means of a donation the Laboratory has been provided with an instrumentarium making it possible to give experimental pathological courses, thus founding the physiological section which no institution of general pathology can dispense with.

But a great deal still remains to be done. In the first place, general pathology is not a special *subject for examination.* Further, the University is still without *an institution* special arranged for the teaching of general pathology.

For a long time the laboratory has been endeavouring to gain these two points so intimately connected. Not till this is done can general pathology be said to occupy its proper place in the curriculum as the main subject round which all the other pathological branches will have to be grouped.

Owing to many causes the present moment appears to be a turning point for the teaching of general pathology in our University.

In other countries also the position of general pathology as a university subject is a burning question. For that reason I think the present a suitable time to place before the public the plan according to which we have been working in this country, and our programme for the future, which, I trust, it will be possible to realize.

The number of universities that can boast of a special chair of general pathology is not great. Of course, lectures on the subject are delivered everywhere, but too often the matter is divided between several teachers conducting the instruction from quite different points of view. Hardly any branch of medical study varies so much as to its limits, or in the way in which it is regarded, as general pathology. This fact strikes the eye at once, when a glance is thrown at the great number of small and large manuals and text-books dealing with the subject. Some, as Bouchard, insert semeiology even as a predominant element; others, as Birch-Hirschfeld, treat the matter from a one-sided anatomical point of view. Cohnheim eliminates the etiological sections altogether. The explanation of this discrepancy must be looked for in the historical development of this branch of study. From a summary of observations, made at the bed side, it has developed into an independent experimental science, that is, *pathological physiology*. As to its rightful position Virchow, when still a young man, spoke the decisive word, as early as 1847, in the „prospectus“ that made the introduction to his „Archiv“: „Die pathologische Anatomie und die Klinik, obwohl wir ihre Berechtigung und Selbständigkeit vollkommen anerkennen, gelten uns doch vorzugsweise als die Quellen für neue Fragen, deren Beantwortung der pathologischen Physiologie zufällt.“ But this „pathological physiology“ cannot nowadays be so easily detached from general etiology as it was in the days of Cohnheim, when bacteriology was still in its infancy.

The discrepancy conspicuous in manuals and textbooks has appeared likewise in the practical teaching of general pathology at the different universities. This is due, in a great respect, to the enormous ground this science has gradually covered, and to its varied technics. Goethe says: „Ein Leibartzt musz zu allem taugen. — Wir fingen bey den Sternen an — Und endigen mit Hühneraugen.“ And matters have become worse since then. It has been getting more and more impossible for one man to master the whole subject. Consequently its division among a greater number of teachers

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has been more readily accepted, and local or personal circumstances can more easily influence the arrangements at the different universities. Two circumstances have proved especially mischievous to general pathology. Firstly, its frequent annexation by morbid anatomy, as the rule is in the Scandinavian countries and Germany, the consequence being that a great stress is laid by the teacher upon the anatomical sections of general pathology at the expense of the physiological sections. Secondly, the annexation of bacteriology by the chair of hygiene, thus doing injustice to the pathology of infectious diseases, removing it from its proper place. This matter I shall return to presently.

At this University the teaching of general pathology has taken quite a special form consisting of nothing but *practical laboratory work* comprised in a *bacteriological*, a *parasitological* and an *experimental pathological* course. No systematic teaching by means of lectures has been organized till now, nor could it be to any purpose in the present state of studies. As long as general pathology is not a special subject *for examination*, any attempt to attract a great number of students to any instruction of the kind is sure to fail. Not that our students are lazy — on the contrary! It cannot be denied that most of them are hard working; but they are on the stretch, rushing from one lecture to another, and in spite of their many years of study — seven on an average — they have no time for anything but what is absolutely necessary for their examination. A small number of lectures and *colloquia* on general pathology I started by way of experiment, did not prove a great attraction.

Matters are quite different when we turn to the laboratory courses,. especially with the bacteriological classes. They were started at a moment when access to the teaching of medical bacteriology was difficult everywhere[2]). From the outset, they were eagerly attended by young doctors; later on, by students as well. When, ten years afterwards, a chair of general pathology was founded, it was practically the chair that was annexed to the laboratory and not vice versa. The result of this, and a happy one, was that the teaching of bacteriology was committed to the hands of the professor of general pathology. This is not, as we know, the case at all universities, medical bacteriology often being entrusted to the professor of hygiene. This remarkable plan originated in Germany. The historical explanation of it must be looked for, no doubt, in the fact that bacteriology, which in Germany had made such an immense progress under the leadership of Rob. Koch, was used as a means of agitation when it was a question of carrying through the foundation of hygienic institutions at the German universities. It may have been very wise, under the circumstances, to make use of that expedient, but there is no reason why such an arrangement should be adopted in other countries. The professor of hygiene must, of course, be able to undertake bacteriological researches, and every hygienic institution is nowadays to be supplied with a fully furnished bacteriological laboratory. But to combine the teaching of bacteriology with that of hygiene would be as unreasonable as to assign the teaching of pathological anatomy to the professor of medical jurisprudence because the latter is bound to master the pathological anatomical technique and to have a post-mortem-room at his institution.

Well then, at this University, owing to special circumstances, the practical instruction in the pathology of infectious diseases became predominant and at last (1900) compulsory. This compulsory

(1) COURSE OF MEDICAL BACTERIOLOGY is given each term to thirty-three students; it lasts six weeks, during which time the students are working at the laboratory from 1 p. m. till 4 p. m. four times a week. This is, in my opinion, the shortest time that can possibly be spent on the subject, if the student is to have practise in general bacteriological technique, become acquainted with the most important clinical bacteriological methods of research, and altogether learn sufficient so as to be able to go on working by himself and to follow and fully understand modern medical literature. And even then it is necessary to put one's shoulder to the wheel if everything shall be achieved within the said time. On the other hand, considering the great many subjects already taught, no further extension of the bacteriological training is possible. Time must be economised, and to make room for the most important subjects it has been necessary to eliminate several sections of applied bacteriology, such as the experiments with disinfectants, which were formerly included, and to restrict to the uttermost minimum the introductory lectures. The tenor of the course is, on the whole, the same as in other places, only less stress is laid on making the students go over a great number of species, and more on their using the material for illustrating general pathology of infectious diseases.

Another position is held by

(2) THE PARASITOLOGICAL COURSE, which was given in the laboratory for the first time in 1901, though, owing to the circumstances, in a fragmentary form only. Since Max Braun in the first edition of his *Parasitenkunde* complained that no helminthological practica were given

at the universities, no such great alterations in this respect have been made as one might have expected considering the number of new animal parasites that have been discovered since 1883. It is rare, in any case, for the medical student to get access to such systematic courses in the science of the animal parasites of man as are given by Stiles at John Hopkin's and Georgetown Universities as parts of his course on medical zoology, or by Raph. Blanchard of Paris, where there is a special chair of parasitology with its appurtenant laboratory. Still rarer is it for the medical student to have the opportunity of going through parasitological practica as Braun thought desirable. And all this in spite of the fact that Animal Parasitology has, of late, come more and more to the front. Only think of all the Sporozoa investigations that have been made recently, more especially the malaria investigations, of the researches into Texas fever, ngana, dysentery, filariosis, etc.

As so often before, special political views have here directed the course of scientific investigations, giving them a new impulse. Indeed, colonial policy has afforded to numerous investigators an opportunity of studying the endemic diseases of tropical climates, giving to their researches the great practical importance which resulted in the establishment of the Schools of Tropical Medicine at Liverpool, London, Hamburg, Bordeaux and several other places. At such institutions the study of animal parasites takes a prominent place[3]); medical work in the tropics requires, Manson rightly says, something more than a smattering of helminthology. The parasitologists, on their side, have made use of the enthusiasm for colonial policy to further their aims. Thus Blanchard, in his periodical, takes this position when agitating in favour of a French institution of tropical medicine, and Stiles refers to the relation of the States to the Philippines[4]) when striving for the introduction of a wider parasitological instruction at the American Universities.

It is beyond all doubt that animal parasitology can boast a development which, under other circumstances, would have amazed the medical world, but which is being overshadowed by the all-eclipsing results of bacteriological researches. The predominance of bacteriology has also, as far as *teaching* goes, been fatal to parasitology. I have tried to redress this wrong by starting a practical course in animal parasitology of a similar description to the bacteriological one. Of course, in a small country, with such limited means as ours, a course of instruction like the one offered at the schools of tropical medicine is quite out of the question. On more than one important point we had to put up with makeshifts using free living amæbæ instead of parasitic ones, the trypanosoma of the hamster instead of the surra and ngana parasite, demonstrating malaria preparations instead of making investigations in vivo, etc. But on the other hand such practical work will afford an opportunity for following some of the fundamental experiments of helminthology, as feeding with trichinæ and possibly with some form of tænia. At these courses special stress is laid upon making the students carry away a collection of preparations to be referred to during their future studies.

Only a restricted number are admitted to each of these parasitological courses. It is to be hoped, however, that it will be possible some day to so increase the accommodation that all students can attend the class. Indeed, I consider such laboratory work in parasitology as of the greatest importance to medical training. The teaching of pathology of infectious diseases is only partly accomplished by means of a bacteriological course; it requires, as a necessary supplement, practical laboratory work with the animal parasites, even though, by a bold extension, *Bacteriology* is made to embrace the sporozoa. Furthermore such parasitological classes will necessarily lead to incursions into the provinces of tropical diseases and *geographical pathology*, and there is every reason to give the students an idea, at least, of these important chapters of animal and vegetable geography, that they have neither time nor occasion to enter into more thoroughly during their studies.

However great may be the value attributed to the two above-mentioned courses, both of which have their starting point in etiology, *pathological physiology* ought to be the central point in the teaching of general pathology.

I have already explained the local circumstances which, at the University of Copenhagen, made it necessary to begin with bacteriological instruction. The external conditions for adding practical work in animal parasites were rather favourable. This was far from being the case with pathological physiology. Both premises and materials were wanting. Being of opinion that it would not be right to put off any instruction of the kind till an Institution for General Pathology had been established I tried to procure the necessary equipment in a private way. I succeeded in making the Board of the „Julius Skrike Fond“ feel an interest in the matter, and by a donation they enabled the Laboratory to obtain physiological instruments sufficient for ten students and

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to get a collection of physiological apparatus and literature. The demand for *special premises* could not be supplied; therefore the bacteriological laboratory had to be used for training students in experimental pathology as well, regardless of the many and great inconveniences that followed.

Even now I dare not propose to make such a course in experimental pathology compulsory at this University. Not that I make light of its importance. On the contrary; the students learn, in this way, how to make experiments. There is no better means for making them understand how difficult it is to account for the causes and development of a morbid state; or how momentous the part of *experimental* investigations has been in interpreting the observations made both clinically and in the post-mortem room. The more or less difficult experiments which they have an opportunity of making, will form a series of „experimenta memorialia“, round which a great deal of book-knowledge will crystallise in their memories. My only reason for not advising the introduction of this course as a compulsory one, is that so many compulsory courses which may be considered of still more importance, already exist at our University. Such a course would necessarily take up a great deal of time and work. We could never ask of our students such intense and comprehensive experimental work as they do at certain American Universities as the Harvard Medical School, where Porter has, in the most admirable manner, organised the study of normal physiology founding it on experimental courses[5]). But even if we are more modest than the American university teachers, it would still be impossible to fit a compulsory course of pathological physiology into the curriculum of our Faculty, as it would destroy the symmetry of the syllabus. It is a different thing when the courses are arranged for *a limited number of voluntary workers.* That should give the more advanced and zealous students, who could find the time for and would be glad of such a course, the feeling that at our University the medical students were still enjoying some of that „freedom in study“ which, on all sides, is laid down as a principle, but can scarcely be said to exist in practise any longer, because of the too many years and subjects demanded by the curriculum.

It is necessary, of course, that when arranging such an experimental course of pathological physiology one should select one of the chief subjects of pathology as a nucleus for practical work, round which experiments taken from various other domains might group themselves. The physiologist will, as a rule, choose the physiology of the nerves and muscles as the main object of this practical work in *normal* physiology, and if we are to go by the elementary text-books on practical physiology [6]) there is apparently unanimity on the subject. But whether one shares this view or not — and I for one do not share it — it is quite certain that when it is a question of a course of *pathological* physiology the choice must be different, and in my opinion there could be no doubt but that the theory of the morbid changes in the circulation and the blood must be the main subject.

To give some idea of the form and extent of the practical pathological course adopted here, I add the following list of experiments which the students are expected to perform:

1. Preparation of loci electi for investigations on the circulation of the frog [web, tongue, lung, mesentery].
2. preparation and investigation of the heart, blood and bone marrow of frogs.

Each student has to produce the following morbid states in frogs and to make microscopical examination of the same in vivo:

3. Venous engorgement and bleeding per diapedesin and per rhexin [tongue, web].
4. Stasis and alterations of vessels (contraction and dilatation) from chemical causes [tongue, web, lung].
5. Thrombosis (red and white thrombi; mechanical and chemical influence) [lung, mesentery].
6. Embolism [lung].
7. Local hyperæmia and anæmia (by section and irritation of nerves, etc.) [tongue, web].
8. Ischæmia and its consequences (inflammation, necrosis) [tongue].
9. Inflammation (produced by cautery, denudation, etc.) [tongue, cornea].
10. Oedema (hydremic plethora, etc. in frogs).
    Some of these experiments are supplemented by demonstrations on warm-blooded animals: for example, experiments on the rabbits' ear, guinea-pigs' mesentery, etc. are made to illustrate inflammation.
11. As an introduction into kymographic experiments with the frog's heart some practical work with normal and poisoned muscle preparations is done [gastrocnemius, tongue].
12. Different cardiographic experiments on a frog's heart under normal and pathological circumstances (poisons, irritation of vagus, etc.).
13. Artificial circulation in a removed frog's heart.

To this is added a series of experiments in pathological conditions of the blood such as

1). Changes of the red corpuscles produced by poisons, burns, anæmia, etc.
2). Hyperleucocytosis and hypoleucocytosis.

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3). Hæmolytic experiments in the living organism and in test-tubes (normal lysins; — lysins produced by immunising; — bacterial lysins; — immune body and complement).

Of the other experiments undertaken at our course, I might point out the following:

1. Observation of regeneration of tissues in vivo [tadpole's tail].
2. Production of various degenerations.
3. Experiments on irritability of monocellular organisms (chemotaxis, electrotaxis, geotaxis etc.).

It is easily seen, that all these three courses point in the same direction, towards *comparative pathology.*

It is now, no doubt, generally accepted that comparative pathology, in the widest sense of the word, is the end of all pathological research, but its great importance has not been recognised till since the middle of the nineteenth century. Heusinger then foretold that we were standing „à la veille de la création d'une pathologie comparée“, but his learned *Recherches de pathologie comparée 1844* were written more from a veterinary and epidemiological than from a general pathological point of view. Heusinger, anyhow, did not seem to have such a clear grasp of the aim and importance of comparative pathology as Fenger, who already in the previous year had treated the same subject with a masterly hand in a short paper written when competing for the post of medical lecturer7). Fenger here deals at great length with the question of how to explain the low standard of comparative pathology, and demands that it shall be able „to show the evolution of the pathological idea through the animal kingdom.“

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During the sixty years since these words were written comparative pathology has advanced with giant strides. The continual progress of veterinary pathology brought a good deal of new matter, but *bacteriology* has done most to widen the range. On the one hand it has, as it were, entirely effaced, as far as infectious diseases are concerned, the line between animal and human pathology, and on the other it has gradually led to a greater variety of animals being used in pathological laboratories. But also many other factors have promoted the development of a comparative pathology. The increasing experimental character of zoology, the important position which in embryology is held by the experiments in the mechanics of development and teratology, the studies of everything connected with the irritability of monocellular animals, and a number of other circumstances have led to a much greater increase in the quantity of pathological material than any one could have dreamt of fifty years ago. Experimental pathology nowadays is looking for its subjects all over the animal and vegetable world. If Fenger could have read Metschnikoff's *Leçons sur la pathologie comparée de l'inflammation,* he would certainly have found them quite in accordance with his ideas.

But if certain parts of pathology allow of such a comparative treatment, the time is still far distant when *the medical student* can expect to be taught comparative pathology in a systematic way; the latter science may perhaps never become an appropriate subject. Still, whenever general pathology is taught, the ultimate end should be pointed out. The three courses described above are perfectly well fitted for that purpose. In fact, when making parasitological experiments the students are working just as much or rather more with diseases of animals than with diseases of human beings; and in the experimental pathology course the animal world is represented from the protozoa to the mammalia.

I have dwelt so long on these laboratory courses because, in my opinion, *practical work* ought to be the chief point when general pathology is taught as a university subject, and because I think that, at most universities, too little attention is paid to this side of the study. Indeed, especially with regard to *pathological* physiology sensu strictiori, no Faculty that I know intimately or from which I have gathered information about curricula has organised a similar practical course as we have done here.

I hope these practical courses will remain as part of the teaching of pathology at our University. However, it would be better if the two of them that naturally go together, were made into one course on the *pathology of infectious diseases.* Further the laboratory work in experimental pathology ought to be gradually *extended* so as to include an increasing number of the different parts of pathological physiology. The task of the teacher will be to select and arrange, among the many diversified experiments, those which are, at the time, fundamental, instructive, and easy to carry out, and to help the students to work into a whole, by means of lectures, *colloquia* and their independent studies, the rich material of experiments and observations which they have gathered at the two courses.

I do not think it necessary to enter more thoroughly into the question of the theoretical training which is to go together with practical laboratory work. I only want to point out that, in my opinion, it is to be given through *examinatoria.* As already stated, the subjects to be taught are general etiology and general pathological physiology. This is also the limitation of the subject accepted by authors like Samuel and Hallopeau. Samuel seems to me to be on the whole the man who, in the most successful way, has made the enormous matter of general pathology accessible to students. But his *Compendium der allgemeinen Pathologie*, Stuttgart 1880, which in its time was too little used and too little appreciated, is antiquated long ago.

It will not even be difficult to draw the line between general pathology as a branch of study and the neighbouring subject, *morbid anatomy.* The teaching of morbid anatomy has long ago taken a fixed form, and is nearly everywhere organised, on Virchow's plan, in postmortem work, demonstrations of postmortem material and practical courses of pathological histology.

The plan of an *Institute for General Pathology* according to the principles stated above is the direct outcome of these ideas. It should contain *two departments.* One of these should be devoted to *pathology of infectious diseases.* In its equipment due consideration should be given no less to animal parasites than to bacteria and fungi. It should be provided both with a *collection* of pathological anatomical preparations to illustrate those *diseases of animals* which the students would have to deal with in their laboratory work, and with the *literary and cartographic works* necessary for the study of epidemiology and geographical pathology. The other *department* — for *pathological physiology* — should, to all intents and purposes, be arranged as a normal physiological laboratory and equipped with *aquariums and terrariums* in the same way as all modern zoological institutions are.

In full conformity with these principles is the sketch of a General Pathological Institute I have worked out, and which is to form part of the great plan of hospitals and medical institutions which has been the order of the day in this country for many years.

I have not, I think, given general pathology too prominent a place in the medical curriculum, nor made it take up too much of the students' time. For many reasons it ought to be in the front rank. It is at the same time a repetitorium of normal physiology and a bond between this fundamental subject and the clinical subjects. It is the natural neutraliser of the many drawbacks resulting from the increase of specialism.

Indeed, the rapid development of specialities has caused a disunion within the curriculum. The boon the patients derive from this specialising and the increase in technical knowledge demanded of the medical man make it necessary for the student to acquire practical knowledge of every speciality. The drawback of this state of things is that the specialist, who is enthusiastic about his subject, will nearly always forget the true standing of *his own* speciality within the whole range of subjects. The Faculty of Copenhagen has tried to meet the just demands of specialists by organising a series of compulsory courses, at the same time protecting students against unreasonable claims by not making the numerous specialities particular subjects *for examination*. But, anyhow, the growing disunion calls for a counteraction, a rallying subject, and no subject is more suitable for such a counteraction than general pathology. Within the circle of medical subjects in the stricter sense of the word, having reference to diseases and their cure, general pathology, and with it morbid anatomy, stand as the only two representatives of *pure na-*

tural science which accompany the students during the last years of their University training.

At all times it has been a difficult task for the medical faculties to keep the balance between the practical and theoretical branches of study, and, beyond all question, for the last generation the practical side has had the best of it. A very extensive technical training is absolutely necessary for the student nowadays if he wishes to become a tolerably good doctor, and it is only natural that this side of the subject should prove very attractive to him on account of its immediate usefulness. This is a good reason why the universities should take care lest the place taken by the natural science subjects should become too subordinate. And it is of still greater importance to lay stress upon the training in natural science when many years' overproduction of doctors is threatening to impair the social standing of the medical men, in spite of the fact that the great bulk of young doctors leave the University with a greater and more solid practical knowledge now than at any other time. It is quite silly, anyhow, to believe that ethics and trade-unionism can help the lame dog over the stile. The means by which the working class has raised its condition, will never be of any good to the medical profession, whose prominent social standing in former days was due to quite other causes than economic ones.

The difficulties I am speaking of here, are not local. They are felt in other countries as well. At least, the concluding words of Krehl's preface to his *Pathologische Physiologie* (1898) seem to point in the same direction. He, too, thinks there is every reason to bring to remembrance the fact that „Wirksamkeit, Bedeutung und Ansehen des ärztlichen Standes in dem gleichen Maasse sinken . . wie der Arzt aufhört Naturforscher zu sein.“

In the above I have been trying to put forward the programme for the success of which I have been working for a great many years. It really looks as if the prospect of its complete realization was not so unfavourable after all. I pointed out above that the two desiderata of most importance were these: the admission of general pathology into the curriculum as a subject for examination, and the foundation of an Institute for General Pathology.

The former is already half realized. Indeed, while this paper was being written, the Royal assent was given to the proposed reform of the medical curriculum which last winter was worked out by the Faculty. According to this proposal, general pathology will not only be admitted into the curriculum, but is to be the object of both a paper and a viva voce examination. Eight years must pass before this examination can be held for the first time, but the Faculty has now recognised in principle the altered position of general pathology.

As regards the Institute, there are fair prospects for its establishment. There is every reason to hope that the plans of the new clinical hospitals and medical institutions will be accepted during the next Parliamentary session, and amongst these is one for the much needed Institute for General Pathology.

One point only it has been necessary to relinquish. The department for applied medical bacteriology into which I formerly hoped I should be able to convert the Sero-therapeutical Department, has been lost to the Copenhagen University. But it has now been resuscitated as the State Serum Institute both on a much larger scale and much better equipped than would otherwise have been the case.

Nor is this department entirely lost. The new Institute has undertaken to assist in the educational work of the University, and through its rich possession of material illustrating the problems of the theory of immunity, the State Serum Institute will always be of importance to *general pathology as a university subject.*

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III.

SVANTE ARRHENIUS AND THORVALD MADSEN

PHYSICAL CHEMISTRY APPLIED TO TOXINS AND ANTITOXINS

The modern theory of solutions has had from the beginning an intimate connection with physiology; the investigations of Pfeffer and De Vries concerning plasmolyse being one of the starting points for the theoretical researches of Van't Hoff. Since then there have been different adaptions of physical chemistry to physiological phenomena. In this respect it will be sufficient to mention the text books of Cohen, Koeppe and Hamburger, giving a general summary of the investigations in this field of work.

It has not as yet been possible to introduce the methods of physical chemistry into the theory of immunity. This is probably due to the circumstance, that the facts at our disposal have, up to the present time been of very unequal value, the methods of investigation being inevitably imperfect. Some of the most prominent investigators of immunity have, as is well known, already tried to interest physical chemists in their experiments; but the researches being nearly exclusively of a qualitative nature were not then sufficiently advanced for the methods of physical chemistry to be applied to them.

To Ehrlich belongs the honour of the first theoretical foundation of the science of immunity, he having, through the labours of many years, worked out exact methods for the quantitative measurement of toxins and antitoxins. When, by these means, we had gained a wide amount of experience of a few toxins, especially that of diphtheria, we had reached a point where we could extend this principle of measurement to others. So long as these measurements could only be practised on living animals, the number had in the nature of things to be strictly limited. It was therefore a great step forward when Ehrlich introduced experiments with test tubes, for the measurement of toxins and antitoxins and discovered bodies, whose reaction was recognizable in vitro. We have just such a body in the tetanolysin[1]), which by simple colorimetric methods can be measured with great exactitude in vitro. It has this great advantage, that it is neutralised by its antitoxin in an apparently similar manner to the diphtheria toxin, hitherto the most thoroughly studied of the toxins.

The tetanolysin must therefore be looked upon as very well suited to quantitative determinations and therefore to physico-chemical studies, as the manner of experimenting in vitro allows variations in the conditions of experiment, to a much greater extent than is the case with living animals. If we take, for instance, the velocity of reaction between tetanolysin and red blood corpuscules or between tetanolysin and antitetanolysin, or the influence of temperature, this could only be investigated with great difficulty if at all in animal experimentation.

The first thing to be done was to procure a large amount of data and of such a kind, that it could be easily accessible for the theoretical calculations used in physical chemistry. A cooperation between two persons could hardly be dispensed with. One was needed to see that the experiments were made systematically from a physico-chemical stand-point; another had to be familiar with the methods of working and the problems of experimental pathology.

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[1]) **Th. Madsen: Ueber Tetanolysin, Zeitschr. f. Hygiene etc. XXXII. 1899.**

As appears from the following, one of us collected during a space of two years a great amount of information concerning the neutralisation of tetanolysin by antitoxin, whereby a conception of this phenomenon was obtained which was different from the previous one. At the same time a great many investigations were made into the hæmolytic action of bases, previously used by Danysz as a help to investigation of hæmolytic toxins[1]), and the curious action was discovered, which salts of ammonia have upon ammonia in that respect. These two phenomena seemed to be very like two corresponding phenomena in physical chemistry — the phenomenon of neutralisation and the action of ammonia salts upon the electrolytical dissociation of ammonia.

Since we had obtained this result it seemed possible for us to continue our work in this direction with the hope of making still further progress. So last summer we made a series of experiments together. The result of these being not quite conclusive, we resumed them this summer. Although we do not claim to have treated exhaustively this exceedingly interesting subject, yet we believe that we can offer a solution of some of the problems connected with it. In the present essay we shall communicate the most important results of our investigations and we hope that in the future, we shall be able to continue our work and increase our experience in this field. Some short papers on the results we have obtained have already been read before the Royal Academy of Science in Stockholm (October 1901) and before the Congress of Naturalists at Helsingfors (July 1902).

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[1]) Danysz: Contribution à l'étude de l'immunité. Propriétes des mélanges des toxines avec leurs antitoxines. Constitution des toxines. Annales de l'Inst. Pasteur 1899.

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Methods of investigation.

As we have already stated, the test tube method permits us, in a comparatively short time, to carry out a great number of experiments.

The red blood corpuscles, which served for our experiments, were emulsionated with an aqueus solution of equal osmotic pressure. As a rule we used horseblood, which was centrifugalised; after the serum was taken away, the corpuscles were shaken up with isotonic solution and again centrifugalised in order to remove the rest of the serum. Of these red corpuscles; which had been so washed out, 25 cc. were added to 1000 cc. of a 0,85 % solution of sodium chloride. Under some conditions this sodium chloride would have had a chemical action upon the bodies to be experimented on. In such experiments a 7,79 % solution of cane sugar took the place of the 0,85 % NaCe solution. In other cases we varied the quantity of the blood.

10 cc. of this blood emulsion already thoroughly shaken were measured into a test tube, to which by means of a pipette, a certain amount of the solution to be examined was added. If the experiment has been successfull a certain amount of red blood corpuscles will be dissolved. The added solution always therefore must contain some hæmolytic body, tetanolysin or alkali, besides other substances which might possibly have an effect upon the hæmolytic process without themselves having a hæmolytic action. For the greater part of the experiments a temperature of 37° C. was used. Then all the test tubes after a thorough shaking were at once put into a water-incubator of the said temperature for 1 hour. They were then taken out and put into an ice safe for about 20 hours. At such a temperature (about 7° C.) the hæmolysis progresses very slowly, and

thus the red blood corpuscles sink to the bottom of the test tube without being further, to any great extent, affected.

This method gives us, as will be shown later on, more complicated results than those methods in which the reaction is suddenly interrupted. The latter was used, when an exact determination of the time of the reaction was required. Then the test tubes, when the reaction was over, were put into a mixture of salt and ice, so that their temperature in a few minutes was reduced to 0° C. After that they were as a rule centrifugalised; this took about 6 minutes.

If we add a large amount of hæmolytic body to the bloodmixture, complete hæmolysis occurs. Starting from a certain concentration upwards, all toxins apparently act in the same manner, total hæmolysis having then taken place (under the category of toxin we include all the hæmolytic acting substances). On the other hand the hæmolysing action decreases rapidly with the decreasing quantity of toxin. If therefore the quantity of toxin is below a certain amount the hæmolysis produced is not recognizable. Quantities of toxin, the action of which would be to produce almost complete hæmolysis or on the other side be very near the limit, where it becomes recognizable, are unsuitable for these investigations. When we used the 2,5 % blood mixture, we obtained the most exact results in the cases where the hæmolysis comprised 4—20 percent of the red blood corpuscles. To this variation in the strength of hæmolysis a variation in the amount of toxin in the relation of about 1 : 3 corresponded. It is thus a comparatively small interval in which the doses, suitable for the experiments, lie.

We proceeded therefore in such a way, that to 10 cc. of bloodmixture different amounts of toxin were added and we aimed at selecting these in such a way that as many as possible of the experiments gave results, which were easily measured. Take for instance from the toxin-mixture to be examined the following amounts 1,0, 0,8, 0,6, 0,5, 0,4, 0,3, 0,25, 0,2, 0,13, 0,12 and 0,1 cc. and add each separately to 10 cc. emulsion of blood. Put all these test tubes at the same time into the incubator and then in the ice safe. The best result is obtained from this series when a hæmolysis of about 10 % occurs in the case of the doses 0,4—0,25 cc. When we know beforehand approximately the amount of toxin with which this occurs, the number of tubes may be diminished, excluding the three first and the three last. In most of the cases, each series included 6 or 8 different doses of toxin.

It would be well, from the beginning, to emphasize that in these investigations errors of experiment occur much oftener than in ordinary physico-chemical experiments with solutions. It may, for instance, happen that a greater amount of toxin has a weaker action than a smaller one. This may be accounted for by the fact that in some cases the added number of blood-corpuscles, through inadequate shaking, was not the same in all the tubes. This occurred in a very high degree in some cases, where the blood corpuscles from some unknown reason showed a strong tendency to agglutinate. When the diluted blood had been standing for some time in the test tubes, before toxin was added, a hasty glance was sufficient to show that in this case the number of red corpuscles differed very greatly in the different test tubes, varying in the proportion 1 : 5. This arose from the very quick sedimentation of these agglutinated corpuscles, so that the blood from this horse must be rejected as unsuitable for our experiments. Usually the corpuscles sink to the bottom, so that a moderate shaking was sufficient to obtain uniform results. Perhaps the above irregularity may be accounted for, that also in the case of not agglutinating blood a smaller number of corpuscles than usual by some accident may have got into some of the test tubes. Other reasons may be added for such errors of experiment as, for instance, the difficulty of measuring small quantities with sufficient exactitude. We therefore avoided as far as possible measuring smaller quantities than 0,2 cc. Were smaller doses necessary, a dilution 1 : 5 or 1 : 10 was used.

Through using many test tubes in one series, it was easy to discover such accidental errors and eliminate their effect. Yet it happened sometimes that a whole series deviated from the normal series. For such reasons it is necessary to have a great many control experiments to avoid mistakes. It is therefore only a small part of our experiments, which are given here.

The experiments have, as a rule, had as their object, to determine the hæmolytic power of various mixtures of toxin. From each mixture of toxin, a series was prepared according to the above method. The different series were treated as uniformly as possible, and after the corpuscles had subsided (during a period of 20 hours in the ice safe) the dissolved amount of blood was estimated through comparing the colours by means of a scale. The colour scale was easily prepared in the following manner: 2,5 cc. red blood corpuscles were added to 97,5 cc. pure water, whereupon complete hæmolysis occurred and a coloured fluid was obtained. The colour strength of this was put at 100. Through diluting it in proportions 1: 2, 1: 4, 1: 5 etc. the degrees of colour 50, 25, 20 etc. were obtained, corresponding to a hæmolysis of 50, 25, 20 % etc. 10 cc. of these dilutions were put into test tubes similar to those used in the experiments. With these fluids of different shades in test tubes, the tubes under experiment were compared, and it was thus shown, how many of the red blood corpuscles had been dissolved. The mixtures of toxin which had yielded the same degree of hæmolysis, were considered to have an equal amount of toxicity. In some cases, for instance in experiments with toxin and antitoxin, where the active toxin was the same, the same toxicity corresponded to the same amount of free toxin pr. cc. In these experiments we have taken into consideration that the amount of fluid varied in the different quantities of mixture of toxin added.

In the first experiments the hæmolysed percentage of blood corpuscles was not determined. We confined ourselves to show the amounts of these different doses of toxin, which had brought about the same degree of hæmolysis, without trying to determining the absolute amount of this.

We must not forget to mention that after a few days the scale of colours must be renewed, because the blood turns violet and is therefore useless for purposes of comparison.

Most of our experiments are carried out by *tetanolysin:* This was prepared 2 years ago by a culture of Bacillus tetani, kindly sent us by Geheimrath Ehrlich. Several broth cultures of this microbe were mixed together and precipitated in the usual way with ammonium sulphate. During these two years, it was found that this toxin got weaker and weaker. The antitoxin used was kindly presented to us by Dr. Libbertz, Farbwerke Meister, Lucius & Brüning, Höchst a/m. The normal serum which we used was usually prepared from horseblood immediately before the experiments took place. The inorganic preparations such as bases, salts and acids were of the best kind which could be procured, and they were tested by comparison with normal solutions.

Experiments with varying quantities of active bodies.

As an example of the above mentioned series of experiments, the following may be cited, in which the amount of blood was (nearly) constant and the amount of toxin varying. The toxin used was partly a solution of 1 part dried tetanolysin to 499 water, which we will call simply 0,2 % lysin — and partly a 0,05 n solution of ammonia. These solutions were added to 10 cc. 2,5 % blood. The result is shown in the following table, where *a* signifies the amount of the solutions added, expressed in cc., *b* the percentage of hæmolysis; *c* and *d* the calculated quantities, whose significance will be indicated below. The lysin had been standing in solution for 6 days (in an ice safe), and had therefore become about 3 times weaker than fresh lysin.

The action of varying amounts of lysin upon 2,5 % blood for 1 hour at 37° C:

a	*b*	*c*	*d*
1,0	45	0,91	7,4
0,8	25	0,74	6,8
0,6	14	0,57	6,6
0,5	7	0,48	5,5
0,45	6	0,43	5,7
0,4	3,5	0,38	4,9
0,35	4	0,34	5,9
0,3	2,5	0,29	5,5
0,25	2,5	0,24	6,6
0,2	1,7	0,20	6,5
0,17	1	0,17	5,9
0,13	0,5	0,13	5,4

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It appears that the hæmolysis increases rapidly with increasing amounts of lysin. The irregular course of the ciphers shows that the errors of experiments are not insignificant. The hæmolysis increases roughly speaking, in proportion to the square of the concentration *c* of the toxin; this is calculated after the formula $c = \frac{10\,a}{10 + a}$, being directly proportional to the added quantity *a* and inversely to the volume $10 + a$. If the square root of *b* be divided by *c*, then the result is nearly constant as indicated under *d*. Their mean would be 6,1, and the deviations from this are so small that they do not go beyond errors of experiment. Three other series of experiments carried out with lysin about the same time showed similar results.

An experiment with 0,05 n ammonia gave the following results:

The action of varying amounts of ammonia upon 2,5 % blood. 1 hour at 37° C:

a	*b*	*c*	*d*
1,0	65	0,84	9,6
0,8	55	0,67	11,1
0,6	37	0,50	12,3
0,5	27	0,40	12,8
0,45	16	0,36	11,1
0,4	12	0,31	11,1
0,35	6	0,27	9,2
0,3	5	0,22	10,2
0,25	1,5	0,17	7,2
0,2	0,7	0,12	6,8
0,17	0,6	0,09	8,3
0,13	0,4	0,05	11,6

The result of these experiments is shown in fig. 1, in which the dotted line represents the ammonia and the straight one the tetanolysin. For ammonia *c* is calculated from the formula $c = \frac{10\,(a - 0{,}075)}{10 + a}$, because the ammonia contained in 0,075 cc. of the added solution is bound by the blood-corpuscles in 10 cc. of 2,5 % blood.

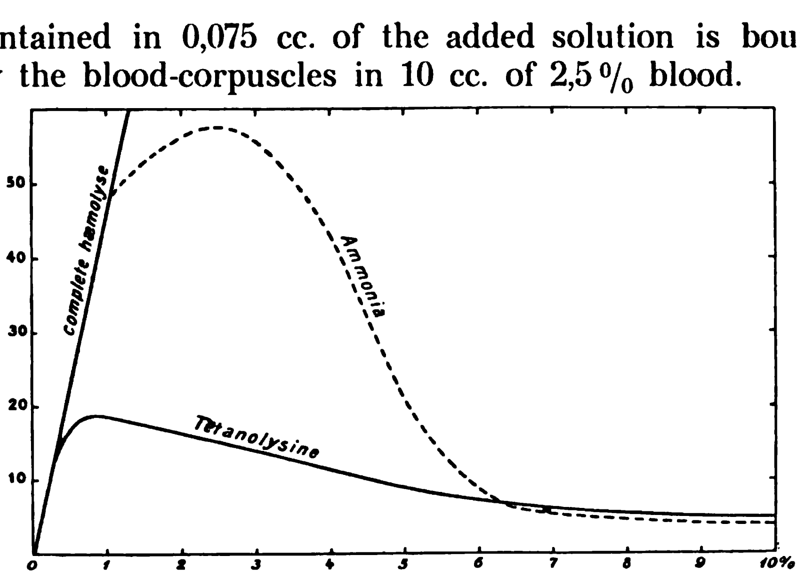

Fig. 1.

This chemically fixed ammonia is in proportion to the amount of blood, as appears from a special experiment tabulated below, which shows the greatest amounts of 1/27 n ammonia and of 1/20 n sodium hydrate which can be added to various amounts of blood without hæmolysis occurring.

Amount of base, which combines with varying amounts of blood:

Amount of blood *a* %	NaOH fixed *b* cc.	*b* (calculated)	NH_3 fixed *c*	*c* (calculated)
20	0,6	0,6	0,82	0,82
10	0,27	0,3	0,42	0,41
5	0,14	0,15	0,19	0,20
2,5	0,09	0,075	0,11	0,10
1,25	0,047	0,038	0,055	0,05
0,62	0,018	0,019	0,027	0,025
0,31	0,011	0,010	0,014	0,013

The calculated amounts af NaOH and NH_3 correspond almost completely to the observed figures and the differences are quite within the limits of errors of experiment. The calculation has been made, under the supposition that the fixed amount is in proportion to the fixing amount of blood. Tha NaOH being 1/20 normal and the NH_3 being 1/27 n, 0,6 cc. of the NaOH solution is equivalent to 0,81 cc. NH_3 solution. It appears from this that equivalent amount of NaOH and NH_3 will fix the same amount of blood. In the case of 2,5 $^0/_0$ of 10 cc. blood and 0,05 n alkali, the fixed quantity is contained in 0,075 cc. of the alkaline solution. The experiment seems to indicate that the combination of a base with the blood corpuscles is of a rather solid character, the ammonia combination not being apparently more hydrolysed than the sodium combination and the dilution having no sensible influence upon the hydrolysis. Yet several facts which will come to light later indicate that such a hydrolysis exists, only not necessarily in such a degree that it is of significance in this case.

In the above calculation it was taken for granted that the ammonia which was fixed to the blood corpuscles had no effect on their hæmolysis but was, so to speak, removed from the reaction. The calculation shows a fairly good agreement with the observed results, the values under *d* being nearly constant. Little notice must be taken of the weak concentrations, where the hæmolysis is below 2 $^0/_0$, for in these cases it cannot be determined with exactitude.

Also the tetanolysin is fixed to the blood corpuscles. This combination seems to be of a less solid character as we did not succeed in finding a sharp limit, where the hæmolysis ceased as in the case of NaOH and NH_3. The figures for tetanolysin have therefore not received corresponding corrections.

One would naturally think that a similar correction should be introduced for the diminishing of the concentration of the blood by the addition af toxin. One might expect that the decrease of concentration would cause a diminishing of the hæmolysis. Yet this does not seem to be the case, because in blood dilution of about the same concentration as the above mentioned, the action is nearly independent of the amount of blood. Therefore no correction in this regard has been introduced above.

This appears from the following experiment, in which the same amount of ammonia (0,6 cc. 0,05 n NH_3) or tetanolysin (0,6 cc. 0,2 $^0/_0$, 8 days old) was added to 10 cc. blood dilution, of which the concentration varied from 0,1 $^0/_0$ to 10 $^0/_0$.

Percentage	Hæmolysis		Percentage	Hæmolysis	
of blood	NH_3	Lysin	of blood	NH_3	Lysin
0,1	5*	5*	2	56	16
0,2	9*	8*	3	56	13
0,3	14,5*	12,5*	4	43	11
0,5	21*	18	6	6	7
0,8	31*	19	8	5	5,5
1,2	50*	18	10	4	5,5

In the beginning, the amount of hæmolysis is proportionate to the added quantities of blood. This happens so long as the hæmolysis is total. In this case an excess of toxin is present, and is therefore able to dissolve the whole quantity of blood and the strength of colour is therefore in proportion to the added amount of blood corpuscles. This occurs in the case of ammonia up to 1,2 $^0/_0$ blood. So 0,1 $^0/_0$ of blood should correspond to No. 4 in the scale of colours, 0,2 $^0/_0$ to 8, 0,3 $^0/_0$ to 12, 0,5 $^0/_0$ to 20 and 0,8 $^0/_0$ to 33. This takes place with ammonia, allowing for errors of experiment. In the case of lysin, the proportionality extends only to the blood concentration 0,3 $^0/_0$, and is nearly right at 0,5 $^0/_0$, so it is probably right also at 0,4 %. The lysin employed is thus only able to dissolve a third part of the blood, which the ammonia solution dissolves. When the blood concentration is increased beyond the limit for complete hæmolysis, the degree of colour increases slightly, but almost imperceptibly; it reaches a maximum when the blood concentration is about twice as much as the greatest amount in complete hæmolysis. Still, the increase in the strength of colour from where hæmolysis begins to where it reaches its maximum, is very insignificant, in fact only 15 %. After this maximum is reached, the colour begin to slowly decrease in strength, more slowly in the case of lysin than in the case of ammonia. The determination of colour becomes more difficult in the case of higher blood concentrations and is therefore rather incertain, for red blood corpuscles cling to the sides of the test tubes and prevent an exact examination.

In the case of a blood dilution of 10 %, f. inst., no perceptible hæmolysis takes place in the ammonia solution, the blood having the property of fixing 0,6 cc. of 0,05 n NH_3. This combination is yet, to a small extent, counteracted through hydrolysis. The corpuscles clinging to the sides of the test tube give a false appearance of depth to the colour. The same applies to lysin, though not to the same extent, hæmolysis always occurring, however strong the blood dilution may be; therefore the colour does not sink so rapidly in this case with increasing concentration of blood. But also in this case blood corpuscles cling to the test tubes and make the colour appear darker than it really is. It is therefore desirable in these experiments not to use too concentrated blood dilutions. About 1 to 2 % are likely to furnish the best results. With too little concentration too little colour is obtained, for the purpose of good measurements.

The fact that, in the case of tetanolysin, a maximum is obtained with certain blood dilutions, indicates that this body also is fixed by the blood corpuscles, as is shown in former experiments[1]) This combination is however probably of a less stable character than that in which bases are combined with blood corpuscles, so that in all cases a perceptible amount of tetanolysin is free, which may have a hæmolytic action. Yet the difference is probably only of a quantitative character.

On this property depends perhaps also another circumstance, which distinguishes the hæmolysis, arising from tetanolysin, from that to which sodium hydrate or ammonia gives rise. In the case of not too strong hæmolysis through bases, after the corpuscles have subsided, two layers are observed in the hæmolysed fluid. They differ very much in depth of colour, the upper one, a few millimeters high, being 2 to 3 times lighter than the lower one. But in the latter too the colour becomes deeper towards the bottom. It would seem as if a sensible time elapsed before the blood corpusles were dissolved by the bases. This might be owing to the small amount of free hydrolysed alkali in the fluid. The blood corpusles gain time to subside to a certain point, before they are hæmolysed, and for this reason the fluid below this point is stronger. The colour of the upper lager may be ascribed chiefly to the circumstance that a collection of red blood corpuscles does not consist of a number of quite uniform elements. A series of these will probably show a very different resistance against hæmolysing toxin1)). The less resistent blood corpuscles will be dissolved a very short time after the toxin is ad ed and before they have subsided.

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[1]) **Th. Madsen: l. c. and: Ueber Heilversuche im Reagensglas. Zeitschrift f. Hygiene etc. XXXII 1899.**

In order to examine the action of varying amounts of blood and toxin, a good many other experiments were made of which some are tabulated below. In the first series 0,8, 0,6 and 0,4 cc. of 1/40 n NaOH or 1,0, 0,7 and 0,5 cc. of 1/40 n NH_3 were added to 10 cc. blood dilution with a percentage, varying from 0,05 °/₀ to 10 °/₀. The experiments with 0,3, 0,2, 0,15, 0,1 and 0,05 percent all gave total hæmolysis, corresponding to the degrees of colour 10, 7, 5, 3,5 and 1,7. They are therefore not included in the following table, which comprises the other experiments. It should be noticed that the degrees of colour are very nearly in proportion to the amounts of blood in the cases of total hæmolysis which is, as it ought to be.

Action of various amounts of blood on the same amount of NaOH and NH_3.

Percentage of blood dilution	0,025 n Na OH			0,025 n NH_3		
	0. 8 cc.	0. 6 cc.	0. 4 cc.	1. 0 cc.	0. 7 cc.	0. 5 cc.
10	4	3,7	2,5	8	8	4
7,5	14	5	2,5	22	10	7
5	33	9	1,5	40	22	8
3	47	40?	2?	40	30	13
1,6	52*	33	18	33	33	27
1	40*	40*	27	30	27	22
0,6	18*	18*	18*	18*	17	15
0,4	13*	13*	13*	13*	13*	12

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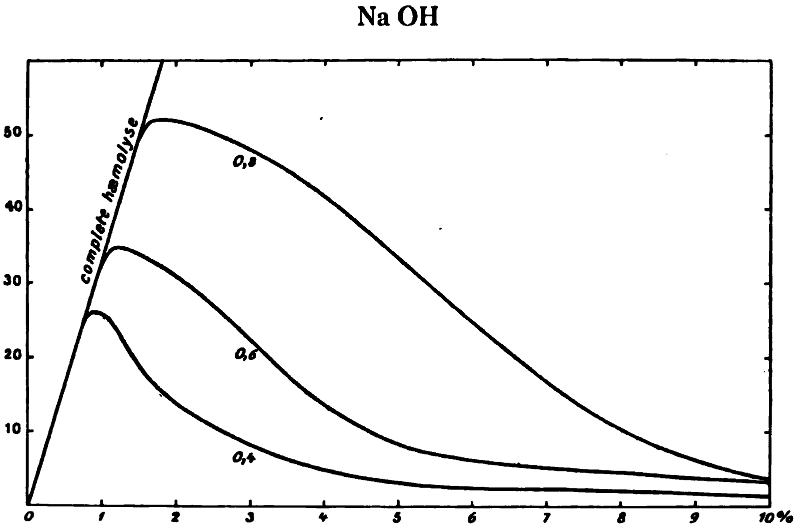
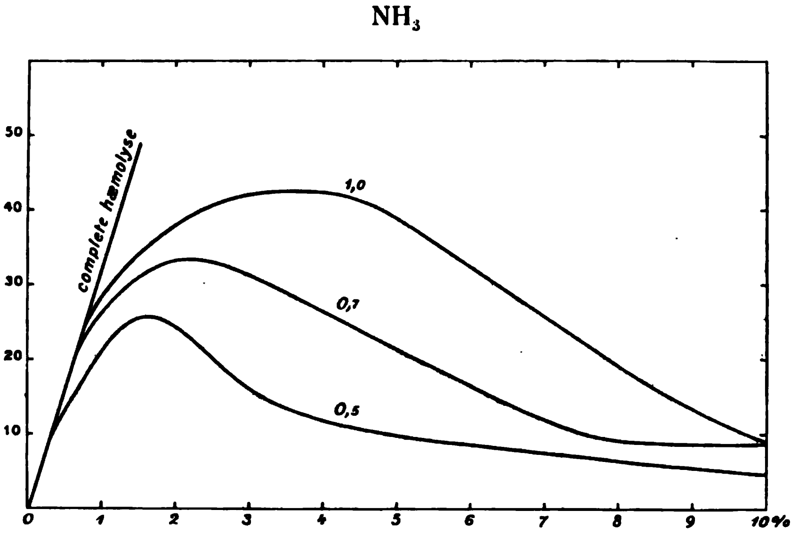
1) Th. Madsen: l. c. p. 218—19.



Na OH

Fig. 2 a.



NH_3

Fig. 2 b.

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The cases where total hæmolysis occurs are signified by an asterisk. To see what would happen with small amounts of blood, when total hæmolysis did not occur, the following experiments were made, in which the amount of alkali was varied together with the amount of blood. In this case 0,01 n NaOH and 0,01 n NH_3 were used.

The action of various amounts of blood upon various amounts of NaOH and NH_3.

Percentage of blood dilution	2	1.5	1.0	0.7	0.5	0.3	0.2	0.15 cc.	
2.0	80 *	37	18	—	—	—	—		0.01 n NaOH
1.2	—	50 *	35	20	—	—	—	—	
0.8	—	—	30 *	23	11	—	—	—	
0.5	—	—	—	19 *	12	1	—	—	
0.3	—	—	—	—	12 *	7	0.5	—	
0.2	—	—	—	—		4	0.25	0.12	
0.1						3.5 *	1.5	0.25	
2.0	45	27	18	—	—	—	—	—	0.01 n NH_3
1.2	—	27	18	10	—	—	—	—	
0.8	—	—	20	11	4	—	—	—	
0.5	—	—	—	12	7	1	—	—	
0.3	—	—	—	—	7	1.5	0.5	—	
0.2	—	—	—	—	—	2	0.25	0.25	
0.1	—	—	—	—	—	3	0.5	0.25	

From these data it appears that a greater (equivalent) amount of ammonia than of sodium hydrate is required, to bring about complete hæmolysis. It also appears that to bring about complete hæmolysis of small quantities of blood a relatively larger amount of sodium is required than for larger amounts. 0,1 % blood dilution corresponds namely to 0,3 cc. of the NaOH solu-

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tion, while to complete hæmolysis of 2 $^0/_0$ blood only 2 cc. solution are necessary, and not 6 cc. as would be necessary in the case of proportionality. Thus the hæmolysis behaves almost as the process of dissolving of different metalhydrates in ammonia or caustic soda. In the latter case a part of the hydrate is precipitated through dilution with water, which indicates that in diluted solution a relatively greater amount of base is required to dissolve a certain amount of hydrate. In the case of hæmolysis the process can naturally only take one direction, an already dissolved blood corpuscle not being able to become a corpuscle again by dilution with water. When a base is added, in a quantity proportionate to the number of blood corpuscles, the hæmolysis extends only to a certain limit, comprising a certain fraction of the blood corpuscles present, and this fraction increases with their quantity, the quantity of water being constant. The process seems in many respects analogous to precipitation through a base of a metalsalt, for inst. aluminium chloride. At the commencement, for the formation of the precipitate, an amount of base is consumed, which is in proportion to the amount of salt, and later on the precipitate is dissolved through a further addition of base, in which case, as above mentioned, the hydrolysis has the effect that the quantity of base necessary for solution of a certain quantity of precipitate increases with the quantity of water present.

In the last tables a great many examples are found indicating that with the same amount of alkali a maximum of hæmolysis always occurs with a certain amount of blood. In the case of sodium hydrate such a maximum is very near the amount which corresponds to total hæmolysis when as much blood as possible is added.

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In the case of ammonia, the difference is considerably greater. The table on page 18 indicates still greater differences than those which ought to occur when we had not used so great intervals in the variations of the amount of blood. Taking the following figures into consideration the value 30, observed in the case of 1 cc. NH_3, acting upon 1 % blood dilution, ought to correspond very nearly to complete hæmolysis, according to the law of proportionality between the degree of colour and amount of blood, in the case of complete hæmolysis.

The difference between the maximum 40 and the maximum value for total hæmolysis in this series, 30, is about 30 %. There is, on the other hand, a much greater difference between the maximum value for 0,5 cc. NH_3, 27, and the maximum value for total hæmolysis in the same series, which exceeds 10 and might perhaps reach 12. The maximum is also much flatter in the series with NH_3 than in those with NaOH as is clearly shown in fig. 2 (a & b) which in a graphic manner reproduces the ciphers from the table on page 18. All these phenomena may be considered as consequences of the fact, that ammonia is a weaker base than sodium hydrate and its combinations therefore are subjected to a higher degree of hydrolysis. It depends on this without doubt, that ammonia which, in the case of smaller blood concentrations, effects less hæmolysis than sodium hydrate does, (supposing that the hæmolysis is incomplete), in the case of higher blood concentrations can have a stronger hæmolytic action than the more powerful base. This seems to signify that also the first combination of base with the blood corpuscles suffers from a perceptible, though very small, hydrolysis.

It appears also from the above tables, that the regularity which was first noticed, of the hæmolysis being nearly proportionate to the square of the quantity of toxin is only correct to a certain extent; that is to say for the blood dilutions, varying from 7,5 % to 10,8 %. In the case of sodium hydrate this regularity is only found in a very small portion of the series. We shall later on return to the explanation of this phenomenon.

Experiments were also undertaken to find the action of bases on blood corpuscles diluted with a solution of *cane sugar*.

The results are given in the following table, in which *a* in cc. signifies the added amount of the base which heads the table. The ciphers in parenthesis belong to an older series of experiments with blood from another bleeding.

a cc. added	1/80 n. KOH	1/80 n. NaOH	1/80 n. LiOH	1/80n. $\frac{Ca(OH)_2}{2}$	1/80n. $\frac{Ba(OH)_2}{2}$
0.5	24 (30)	20 (30)	15 (30)	5 (5)	5 (5)
0.4	9 (17)	8 (17)	7 (15)	3 (5)	4 (5)
0.3	5 (3)	6 (5)	6 (7)	2 (5)	3 (4)
0.25	3.5 (3.5)	3 (3)	3 (4)	2 (5)	2.5 (4)
0.2	2 (2)	2 (2)	3 (1.5)	2 (4)	1.2 (3)

Allowing for errors of experiments, equivalent amounts of the three alkalies give similar values. But the action of the hydrates of the alkaline earths is much smaller (excepted perhaps the case of very diluted solutions). This seems to rise from the fact, that on the test tubes with calcium and baryum a precipitate is formed, which seems to consist of faint red crystal needles. The product of hæmolysis seems in this case not to be so easily dissolved as that of alkalies. The hæmolysis is therefore nearly constant, and would probably be yet more so, if it were not for the presence of the red needles which partially clinging to the wall of the test tube give the fluid a fictitious depth of colour.

In the older series the measurements were made by comparison with the upper part of the hæmolysed fluid, and therefore the numbers within the parenthesis are nearly constant.

As the above data show it is characteristic of the strong monovalent alkalies that their hæmolysis increases very quickly with the amount of alkali, and this increase is at a greater rate than it should be, if it were proportionate to the square of it. This phenomenenon depends without doubt on the comparatively small hydrolysis of their combinations with the blood corpuscles.

We have still to give a more detailed report concerning the action of various amounts of blood on the same amount of toxin. The results of these experiments are shown in the following table, in which the quantity of toxin varies from 1,6 cc. to 0,05 cc. of a 0,1$^0/_0$ solution. The arrangement of the table is the same as in the previous one. The experiments are made on blood corpuscles, diluted in salt solution at 37^0 C for 1 hour.

The maximum value, which in this case is very little pronounced viz. flatter than in the experiments with ammonia — occurs in general with a smaller amount of blood than for the alkalies. In the case of the solutions which contained the most toxin (1,0 and 0,6 cc.), the maximum is found with about 6,5$^0/_0$ blood dilution; with smaller amounts of toxin, (0,4 cc. to 0,25 cc.), it is found with about 0,2 $^0/_0$ blood dilution and for the weakest solutions of toxin (0,08 cc.) first with 0,1 $^0/_0$.

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Amount of blood	1.6	1.0	0.6	0.4	0.25	0.15	0.08	0.05
10 per cent	8	4.5	„	4	2?	„	„	„
5 „	11	4.5	2.5	2.2	1.5	1.3	„	„
2 „	11	6	4.5	3	„	„	„	„
1 „	14	7	5	4	1.5	1	„	„
0.5 „	„	12	4	2.2	0.7	0.4	„	0.2
0.3 „	„	7	4.5	2.5	1.7	0.7	0.4	0.2
0.2 „	„	6*	4	3.2	2.2	1	0.4	„
0.15 „	„	4.5*	3.5	3	2.2	1.5	0.5	0.2
0.10 „	„	3*	3*	2.5	2.2	1	0.6	„
0.05 „	„	1.5*	1.5*	1.5*	1.2	1	0.5	0.2

The combination between toxin and blood corpuscles must therefore be regarded as still weaker than between ammonia and blood corpuscles. The most obvious standpoint from which to consider these facts is the following. For the combination of toxin or lysin with the corpuscles a certain amount of time is required. This is comparatively small in the case of sodium hydrate. If then a quantity of blood, too large to allow complete hæmolysis, is added, the sodium hydrate is fixed to the blood with comparative quickness and only the surplus is free to hæmolyse the combination blood-alkali. The hæmolysis therefore reaches the maximum of colour at about the point where complete hæmolysis occurs. First in diluted solutions, where the reaction is slower and the hydrolysis stronger, the sodium hydrate gains time to hæmolyse some of the alkali-bound corpuscles, before it is fixed to other blood corpuscles.

The maximum occurs therefore with a larger amount of blood than that which corresponds to complete hæmolysis. This is yet more the case with ammonia; this has a considerably smaller velocity of reaction than sodium hydrate has. And the tetanolysin which combines with the corpuscles at a still slower rate, has a straighter curve than ammonia. With lysin there is therefore no sharply defined point at which a strong hæmolytic action suddenly takes place.

As in the case of sodium hydrate, the amount of lysin, required for complete hæmolysis, increased slower than it would have done if the increase had been proportionate to the increase of blood. While for complete hæmolysis of 0,5 % blood 0,4 cc. solution of lysin is required, for 0,2 % blood only 1 cc. (instead of 1,6 cc.) was needed and for 2,5 % blood only 3 cc. (according to another series of experiments). The quantity of toxin required for complete hæmolysis increases, roughly speaking, in proportion to the square root of the amounts of blood.

The velocity of reaction of hæmolysis.

To penetrate a little further into the many curious phenomena connected with the process of hæmolysing, a series of experiments was undertaken, dealing with the velocity of reaction or, more correctly speaking, with the time, in which hæmolysis proceeds to a certain degree. For this object we used two different methods. According to the first one, the lysin should during a fixed time, and at a fixed temperature, act upon a blood dilution 2,5 %. Afterwards it should be cooled down, centrifugalised and the strength of the hæmolysis determined through the colour of the fluid. In this case much stronger solutions of toxin can be applied than in the usual case. We will explain the second method later on.

In this case the surplus of toxin is so great, that it may be considered as practically constant throughout the whole process. As stated above, 10 cc. of 2,5 %

blood can fix 0,04 cc. of a 0,1 n NH_3 or NaOH solution. The amounts used in these experiments will, as is shown below, be much greater, so that without great errors, the active amounts of base may be considered constant. In all cases in the calculation, the said amount has been substracted from the amount added.

But on the other hand that quantity of blood which has not been hæmolysed, decreases during the reaction. It is natural to suppose that the amount transformed per unit of time, is proportionate to the amount of blood corpuscles present. If therefore the number of these arbitrarily be fixed at 100 to begin with, and at a given time at 100 — x, the following differential equation results:

$$\frac{dx}{dt} = K\ (100 - x)$$

or integrated, where t signifies time

$$\log \frac{100 - x_0}{100 - x_1} = K_1\ (t_1 - t_0)$$

All the calculations of velocities of reaction, determined by means of the first methods have been made according to this equation. In these calculations the ordinary logarithms have been used, so that K is 2,3025 times greater than K_1. As an example some determinations of the velocity of the reaction of ammonia at 37° C may be quoted.

A. 0.75 cc. $^1/_{30}$ n NH_3	t	13	27	51	82 min.
	100—x	97	89	62	25 perc.
	K_1	0,0010	0,0019	0,0047	0,0073

B. 0.5 cc. $^1/_{10}$ n NH_3	t	6	14	23	31 min.
	100—x	97	82	60	35 perc.
	K_1	0,0022	0,0062	0,0096	0,0147

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C. 1 cc. $^1/_{10}$ n NH_3	t	5	9	15	19 min.
	100—x	91.5	80	57	44 perc.
	K_1	0,0077	0,0107	0,0163	0,0187

D. 2 cc. $^1/_{10}$ n NH_3	t	2.25	6.2	8.2 min.
	100—x	88	70	59 perc.
	K_1	0,026	0,025	0,028

It appears from these examples that K_1 usually increases very rapidly during the process. Only in the last case K_1 is nearly constant and this is probably owing to errors of experiment. It is easy to imagine, that this increase of the velocity of reaction, as time goes on, depends on the circumstance, that the red blood corpuscles' cellular membrane must be destroyed before hæmolysis can occur. In the beginning no hæmolysis at all takes place. Little by little the most accessible alkali or lysin-bound corpuscles are hæmolysed, and later on the most resistent, whose membranes will be destroyed only after a prolonged action of the toxin. In chemistry several similar phenomena occur, especially when solutions act upon solid bodies; and it is then said, that a certain time of induction is necessary, before the attack occurs. Physicists often find it difficult to understand what this time of induction means. In the present case it is easily explained why there should be a time of induction, this being here equivalent to the time required for the destruction of the cellular membrane.

In order to clear up the connection between the concentration of toxin and the velocity of reaction, we must therefore proceed so that we ascertain if a certain dose of toxin produces the same degree of hæmolysis during a certain time as half that dose of toxin effects in double that time. From the above figures this would seem to be, roughly speaking, the case. A correction must be introduced here, just as above, (p. 12) for the change of volume through the addition of toxin. In this case, after we have supposed that the reaction proceeds in proportion to the quantity of blood also, the correction should be applied as well to the calculation of the concentration of the blood as to that of the lysin. If the concentration and the velocity of reaction are proportionate to one another, in the present case the times, in which the same degree of hæmolysis is obtained, should not be in inverse proportion as in 1:2, 4:8 but as $(1:1.075^2):(2:1.05^2):(4:1.10^2):(8:1.20^2) = 0.865:1.814:3.345:5.554 = 6.4:3.06:1.66:1$

Through the correction for the quantity of fixed ammonia, these ciphers will be changed to 7.64 : 3.33 : 1.73 : 1.02 = 1 : 0.44 : 0.23 : 0.133.

Through simple interpolation from the above ciphers it is found that the following degrees of hæmolysis require the times mentioned below to become hæmolysed:

x	3	10	20	30	40 percent
A	13	26	35	44	53
B	6 (5.7)	10 (11.5)	15 (15.4)	18 (19.4)	23 (23.3)
C	„	5.5 (6.0)	9 (8.0)	12 (10.1)	14 (12.2)
D	„	1.8 (3.5)	4 (4.7)	6.2 (5.9)	8 (7.1)

In parenthesis ciphers are quoted, which are calculated under the following suppositions: proportionality between hæmolysis and the amounts of ammonia — and that the observations in the first series (A) are right. The concordance between the calculated and observed values is very good, allowing for the great errors of experiment. The greatest deviations are found between the first figures in the D series. It ought not to surprise us that rather important errors in the determina-

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tion of the times can creep in, owing to the time taken by the cooling process.

In the case of sodium hydrate and x=6 the following times 20,5 , 9,8 and 4,2 minutes are found for the addition of 0,15 , 0,3 and 0,6 cc. of 0,5 n NaOH at 37^0 C. The calculated figures are (20,5), 10,2 and 5,3. The concordance is satisfactory.

What is said for ammonia will also apply to lysin. The rate of reaction increases with the amount transformed.

In the table below, the results of some experiments at 20^0 C are quoted. The signs are the same as those used above in the ammonia table.

		x = 7	13	20	30
A	10 cc. blood + 0.3 cc. 0.2 $^0/_0$ lysin	11	17	25	„
B	do. + 0.6 „ „	5 (6.8)	7.5 (9.0)	10.2 (13.2)	15.5
C	do. + 1.0 „ „	„	4 (5.8)	5.9 (8.5)	9.2 (10.0)

Supposing that no lysin is fixed by the corpuscles, (a supposition which is certainly not true) the times ought to be in proportions of 1 : 0.53 : 0.442. As appears from the values in parentheses (which are calculated in this way) the reaction proceeds quicker at the higher concentrations than it would if regulated by proportionality. The deviations are yet not so great, as not to allow some doubt about the validity of the law of proportionality in this case. We should be more correct in supposing that a part of the lysin is fixed by the blood corpuscles. But still also in the case of ammonia and sodium, greater amounts act somewhat quicker than is required by the law of proportionality. This deviation probably depends on the method of experimentation.

As well this circumstance as the chemical fixing are effective in the case of lysin, therefore the deviation in this case is greater than with the alkalies.

We can now get an idea of the causes of the above mentioned rule, that the amount of blood hæmolysed is proportionate to the square of the amount of toxin (within certain limits). One might perhaps at first expect thas this would indicate that the velocity of reaction should be proportionate to the second power of the amount of toxin, while we have just found that it was proportionate to the first power.

Let us take for example the series C in the case of ammonia. From this it appears that a proportionality between the velocity of reaction K_1 and the square root of the amount transformed, x, takes place.

The following are the values:

t =	5	9	15	19 min.
x =	8.5	20	43	56 %
$K_1 =$	0.0077	0.0107	0.0163	0.0187
$10^3 K_1 : \sqrt{x} =$	2.64	2.40	2.49	2.50

Sodium hydrate (0,3 cc. of 0,05 n solution to 10 cc. blood dilution) gives at 37° C the following values which, if they do not follow the said rule as closely as those in the table given above, still follow it, if we allow for rather great errors of experiment.

t =	17	22	27 min.
x =	7	18	35 %
$K_1 =$	0.00185	0.00392	0.00693
$10^3 K_1 : \sqrt{x} =$	0.70	0.92	1,17

In the case of tetanolysin (0,6 cc. 0,2 % lysin) at 20° C the following values are observed and calculated:

t =	5	9	11.5	15 min.
x =	7	17	24	30 %
$K_1 =$	0.0063	0.00899	0.01036	0.1034
$10^3 K_1 : \sqrt{x} =$	2.38	2.18	2.12	1.89

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All these series agree with the above rule, allowing for errors of experiment. (To avoid mistake we mention here, that a great many series, which will be dealt with below, deviate from this rule).

Is it not by accident that the constant K_1 in the sodium series increases quicker than in proportion to $\sqrt{x}$, while ammonia follows closely this rule, and lysin shows a deviation in the inverse direction as NaOH (see p 15).

If the rate of reaction at each moment were proportionate to the square root of the amount transformed, then in the case of small values of x, when 100—x could approximately be considered as constant, the velocity of reaction would be expressed by the equation.

$$\frac{dx}{dt} = K\, a \sqrt{x}$$

in which a stands for the quantity of toxin; from this follows

$$\sqrt{x} = 2\, Kat$$

the amount transformed should then be proportionate to both the square of the time of reaction and to the square of the amount of active toxin. Both these relations are only approximately right, especially the first. This gives for the three last mentioned velocities of reaction the following values for $t : \sqrt{x}$.

					mean
ammonia	1.7	2.0	2.2	2.5	2.1
sodium	6.4	6.4	5.2	4.7	5.7
lysin	1.9	2.2	2.4	2.7	2.3

In all three cases variations from the mean of about 20 percent occur. At all events these rules may have a certain value for the purpose of interpolation.

This furnishes a good example of a velocity of reaction which examined during a certain time, (1 hour), seemed to be proportionate to the square of the active amount of toxin, but which is in reality proportionate to the first power of it. We thought this case of sufficient interest to justify the thorough analysis given above.

The *influence of temperature on the velocity of reaction* is an interesting question. In ordinary chemical processes, the velocity of reaction increases for each 10° C. with values lying between the proportions 1.93 : 1 (in the case of saponification of ethylacetate by strong bases) and 4.2 : 1 (in the case of inversion of cane sugar).

In these experiments it was absolutely necessary to see that the same sample of blood was used for the higher and lower temperatures (2,5 % blood was used). The following data show the increase of the rate of reaction.

Hæmolysing substance.	Intervals of temperature.	Increase.	Increase for 10° C.
0.5 cc. $^1/_{10}$ n NH_3	$3{,}5^0 - 37^0 = 33{,}_5{}^0$	29.8 : 1	2.76 : 1
0,5 „ $^1/_2$ n NaOH	$22{,}_4{}^0 - 37^0 = 14.6^0$	4.4 : 1	2.76 : 1
0.2 „ 0.2 % lysin	$20^0 - 37^0 = 17$	6.6 : 1	3.04 : 1

The figures show that the increase is the same for ammonia and sodium hydrate, just as one would expect. For tetanolysin it is a little greater. All three values lie within the limits of the values for other chemical reactions with which the hæmolysis ist herefore related also in this respect.

Another matter of interest is the *relative velocity of reaction of sodium hydrate and ammonia.* If it had been the hydroxylions which affected the blood corpuscles, one would have expected that a certain amount of sodium hydrate would have acted much more quickly (in the dilutions used about ten times) than an equivalent amount of ammonia. Now this is so far from being the case that, on the contrary, ammonia acts more than twice (2,24) as quickly, three different experiments showing the proportions 1,93, 2,00 and 2,78 in the case of 0,01 n solution. In this concentration sodium hydrate contains about 20 times as many hydroxylions as the equivalent amount of ammonia. This shows clearly that in this case *it is not the hydroxylions which are active.* To the same conclusion we are also led by the fact that the action of ammonia is in proportion to its concentration and not to its square root, the same occurring with sodium hydrate. Probably, in this case, dissociated, as well as undissociated molecules, contribute to the hæmolysis.

Most experiments in hæmolysis have been made by subjecting the tubes to a temperature of 37° C. for 1 hour and afterwards leaving them to cool in an ice safe for about 20 hours. We have therefore found it well to study the influence of time on experiments orranged as above. As appears from the above example, the velocity of reaction in the ice safe (temperature 6°—7° C.) is about $^1/_{20}$ of what it is at 37° C. But during a few hours the blood corpuscles subside and by that means avoid further hæmolysis.

In the following experiments the test tubes were therefore allowed to stand a certain time in the incubator, at 37° C. and were then put into a freezing mixture, in which they quickly cooled down to about 0°; afterwards they were put in an ice safe for about 20 hours. The headings, given below, indicate the time during which the test tubes were subjected to 37° C. Total hæmolysis corresponds in this case to 60.

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Amount.	0 min.	5 min.	10 min.	20 min.	40 min.	100 min.	200 min.
$^1/_{20}$ n. sodium hydrate.							
1.0 cc.	56	60*	60*	60*	"	"	"
0.7 „	55	60*	60*	60*	"	"	"
0.5 „	47	56	60*	60*	60*	"	"
0.4 „	33	53	53	50	60*	60*	60*
0.3 „	22	33	40	40	40	47	60*
0.25 „	"	"	"	"	27	37	30
0.2 „	7	12	12	11	18	20	25
0.15 „	"	"	"	"	5	7	11
0.1 „	"	"	"	"	"	1.5	2
$^1/_{20}$ n. ammonia.							
1.0 cc.	47	53	60*	60*	"	"	"
0.7 „	33	47	60*	60*	"	"	"
0.5 „	27	40	53	47	60*	60*	"
0.4 „	22	40	47	40	53	49	60*
0.3 „	17	33	30	30	48	40	40
0.25 „	"	"	"	"	30	30	28
0.2 „	4	10	12	11	18	14	19
0.15 „	"	"	"	"	7	6	6
0.1 „	"	"	"	"	"	1.5	2.5
0.5 % tetanolysin.							
1.0 cc.	15	24	30	"	"	"	"
0.7 „	11	16	18	"	"	"	"
0.5 „	6	9	9	11	"	"	"
0.4 „	"	6	8	9	11	"	"
0.3 „	2	6	6	7	9	"	"
0.25 „	"	"	"	"	9	27	31
0.2 „	"	3	7	5	8	19	24
0.15 „	"	"	"	6	10	15	18
0.1 „	"	"	"	4	11	7	11
0.05 „	"	"	"	"	"	5	8

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In these tables we do not find the rapid increase of hæmolysis in proportion to the time taken, as in the preceding experiments. This depends of course on the long time passed at the lower temperature, after the time spent at high temperature. These experiments, therefore, do not give such a clear idea of the process of hæmolysing as the last. But they are rather useful for the right conception of the results of the usual method of experimentation. The length of time devoted to heating the tubes has a much greater influence on the action of tetanolysin than on that of bases. And between these the length of time has more influence on ammonia than on sodium hydrate. This seems to indicate that it is the hydrolysed part of the combination of the three lysins with the blood corpuscles, the action of which increases with the rise of the temperature. Probably its hydrolysis increases with increasing temperature and this, in the highest degree, is the case with the combination most easily hydrolysed — that of tetanolysin, and in the smallest degree in the case of the most stable combination — that of sodium. The greatest relative action of the temperature is found in the case of slight concentrations of tetanolysin.

Action of foreign bodies.

Foreign bodies usually have an effect upon hæmolysis; in most cases the effect is weak. In some cases, on the other hand, it is exceedingly strong as, for instance, in the case of normal serum acting upon tetanolysin and in the case of salts of ammonia acting upon ammonia. This action being partly of a great practical significance and on the whole very important for the understanding of hæmolysis, we have tried to find such cases, even when they were not very obvious.

It is to be noticed, that the addition of salts increases the sedimentation of the blood corpuscles; this apparently lessens the hæmolytic action. For this reason, also on this point, several control experiments have been made through determination of the velocity of reaction; these have on the most important points confirmed the results which have been obtained in the usual way (see below).

To begin with, we will investigate *the action of salts upon bases.* In these experiments the blood must be free from salts: it was therefore emulsionated in a sosution of cane sugar. It was obvious from the first experiments, that blood is hæmolysed much more strongly by bases in sugar solution than in salt solution. And certainly salts decrease the action of bases, as will be seen from the following experiment. The solutions of bases, which were added, were all $^1/_{80}$ n in respect to base, but contained various amounts of salt. The first column in each series indicates as usual the amount of solution, added to 10 cc. blood dilution. The second column indicates the degree of hæmolysis.

Amount added	Na, O	Na, 2C	Na, 10C	Na, 50C	Na, 10S	Na, 50S
1.5 cc.	100*	„	100*	90	100*	75
1.0	100*	„	90	27	90	60
0.8	90	„	79	18	79	8
0.5	79	33	12	3	12	3
0.4	71	30	8	2	4	„
0.3	12	6	3	1	„	„
0.25	6	3	2	1	„	„
0.2	3	2	1	1	„	„

The headings indicate: Na,0, that pure $^1/_{80}$ normal sodium hydrate was added; Na,50 C, that the base added was $^1/_{80}$ n in respect to sodium hydrate, but besides was 0,5 n in respect to NaCl. 10, C and 2, C indicate 0,1 and 0,2 normal chlorid. 50, S and 10, S 0,5 and 0,1 normal Na_2 So_4.

In the following tables the headings have the same signification; and for reasons easily understood, to each base only salts of the corresponding metal were added.

Amount added.	K, 0	K, 2 C	K, 10 C	K, 40 C	Li, O	Li, 10 S	Li, 50 S
1.5	100*	„	100*	90	„	100*	90
1.0	100*	„	90	49	„	90	49
0.8	90	„	60	18	„	90	„
0.5	77	60	18	4	85	20	11
0.4	70	28	7	2	63	7	„
0.3	10	6	6	1	17	6	„
0.25	6	3	3	1	8	„	„
0.2	3	1	2	1	3	„	„

The three bases, potassium, sodium and lithium may, when equivalent amounts are taken, be considered quite similar as regards action, allowing for errors of experiment; and likewise all the salts used in the experiments have the same power in lessening the hæmolysis when added in equivalent amounts. This depends most probably on the fact that these bases on the one hand and their salts with the same acids on the other, are dissociated to very nearly the same extent. The action of the salts is still perceptible, although rather slight in such small doses as 0,02 n which when diluted by 50 times as much blood mixture, corresponds to a 0,0004 normal salt solution — when most diluted. In all cases, even in the most diluted salt solutions, 1,6 times as much salt as base is present, according to equivalents. A solution of 0,0008 n salt (addition of 0,04 cc. of 0,02 n salt solution) seems to diminish the action of alkali to the extent of about 20 percent, (viz. the action of alkali is decreased to 80 % of its former strength). 0,004 n salt (0,04 cc. of 0,1 n sol.) decreases the action by about 40 % and 0,02 n (0,4 cc. of 0,5 n salt solution by about 60 % — all of 0,0003 n base.

The action of salt increases not by any means proportionally to the amount added, but much less rapidly, corresponding to about the cube root of it. The action is very similar to the decrease of the degree of dissociation of salts, which takes place with the further addition of salts that is by increased concentration; but it is much greater. It is about what we should expect, according to the rule of Ostwald, would be the case for the degree of dissociation of the salts of very polyvalent acids.

Amount cc.	NH_3, O	NH_3, 4S	NH_3, 10S	NH_3, 50S	NH_3, 10C	NH_3, 50C
2.0	"		79	6	49	7
1.5	"		70	3	49	6
1.0	100*		41	2	41	2
0.8	90	"	27	1	37	2
0.6	"	"	9	"	27	1
0.5	79	60	6	"	3	
0.4	52	24	5	"	1	
0.3	24	16	3	"	"	
0.25	12	10	2	"	"	
0.2	4	"	"	"	"	

Still stronger is the action of salts of ammonia upon ammonia, as the following figures show.

The action of sulphate and chloride, in equivalent amounts, may be considered as the same, allowed for errors of experiment. The degree of dissociation of these salts is also very nearly the same at such high dilutions as are here used. Though it would seem as if the sulphate has a weaker action than chloride in the concentration 0,1 n, yet, in the case of 0,5 n solutions, rather the opposite takes place, and the difference must then, without doubt, be attributed to errors of experiment. Through these series we get a good idea of the possible extent of these errors of experiment and how necessary it is not to consider single figures by themselves, but to take the whole series in consideration. Therefore, in the following, we use the mean values for sulphate and chloride. The action of very diluted solutions of salts of ammonia, 0,0016 n, (add. of 0,4 cc. 0,04 n salt solution) is not stronger ($25\ ^0/_0$) than the corresponding solutions of the salts of the strong alkalies, the series with 0,04 n $N_2H_8\ SO_4/2$ indicates this. When we use one part of 0,1 n salt of ammonia in 6 parts of blood mixture (2 cc. to 10 cc. blood emulsion) —viz. a 0,016 n solution— the hæmolytic action is very much lessened; it is then no greater than that of a 4 times smaller amount of ammonia. If we add half this amount of ammonium salt (0,008 n = addition of 1 cc. 0,1 n salt solution), the action is decreased to about $^1/_3$, and when we add $^1/_4$ of this amount of salt, (0,004 n = 0,5 cc. 0,1 n salt sol.), the action is decreased to about $^2/_5$. By an addition of 0,06 n salt (1,5 cc. 0,5 n salt solution), the action is decreased to $^1/_7$.

We can sum up these values as follows:

S signifies the added salt of ammonia.

S.	0,0016	0,004	0,008	0,016	0,06
reduction	0.75	0.4	0.3	0.25	0.14

The action is of about same character, but at higher concentrations considerably stronger than that of the salts of the strong alkalies, still it is much smaller than the action of ammonia salts upon the strength of ammonia. In this case the largest addition of salt would decrease the degree of dissociation in the case of $^1/_{880}$ n ammonia to about $^1/_{200}$; as may be calculated from the constant of dissociation in the case of ammonia at 25^0 C ($K = 23 \cdot 10^{-6}$).

A dissociation constant of the size $K = 2 \cdot 10^{-2}$ resp. $4 \cdot 10^{-3}$ would be required to obtain a decrease corresponding to the above mentioned in the case of the combination of sodium hydrate and ammonia with blood corpuscles. Constants of this magnitude belong to the chlorophtalic and trichlorlactic acids which are among the strongest organic acids.

The *salts have also an action,* though not a strong one, *on tetanolysin.* This appears from the fact that the action of tetanolysin is stronger in salt than in sugar solution. Still small doses have no perceptible effect, as appears from the following example. The experiments were carried out by adding to tetanolysin sodium chloride and sodium sulphate (in 0,9 and 0,2 n solutions) and ammonium sulphate (in 0,75 n and 0,2 n solutions). The action of the weaker doses is imperceptible, the differences being smaller than the errors of experiment. The action of equivalent amounts of the three salts is nearly similar and amounts to about 50 % in a 0,04 n solution. A salt solution of the strength 0,01 n or less has no or a very little influence. The strength of the lysin in the added solutions was 0,2 % (= addition of 0,5 cc. 0,9 n. salt solution to 10 cc. blood emulsion).

III

Amount cc.	Lysin pure	Lysin +0.9NaCl	Lysin +0 2NaCl	Lysin+0.9 $Na_2SO_4/2$	Lysin+0.2 $Na_2SO_4/2$	Lysin+0.75 $N_2H_8SO_4/2$	Lysin+0.1 $N_2H_8SO_4/2$
1.0	15	25	13	27	12	„	„
0.7	10	17	8	24	7	„	„
0.5	5	10	5	16	5	8	7
0.3	2	5	3	6	5	4	2
0.2	1	3	2	4	4	2	1

It is curious that the hæmolytic action of the three lysins when mixed is weaker than it would be if calculated from the mean. This applies especially to mixtures of tetanolysin and sodium hydrate. In the following series, to 10 cc. 2,5 % blood, diluted with sodium chloride, similar amounts of 0,1 % tetanolysin, $^1/_{60}$ n NaOH and $^1/_{60}$ n NH_3 and of mixtures of these three solutions were added (50 % of each).

Amount cc.	A. lysin	B. NaOH	C. NH_3	$D=\frac{A+B}{2}$	$E=\frac{A+C}{2}$	$F=\frac{B+C}{2}$
1.0	14	22	22	5	8	8
0.7	9	18	10	4	3	6
0.5	5	6	5	2	3	3
0.4	3.5	1	2	1	2	3
0.3	1.7	0.5	1	1	1.5	2
0.2	1	0.3	0.5	0.5	1	1.5

The values in the series D, E and F are considerably lower than the values for both components, if we except the last three quite unreliable values in the F series. Similar experiments with blood, diluted with sugar solution, had similar results. In the mean, the hæmolytic action is almost of the same power, as the sum of the hæmolytic actions of the two hæmolytic bodies, if they were alone in the solution. The first value in the D series is not greater than in the case of the half concentration of sodium hydrate or tetanolysin by themselves or, in other words, the action is just as great as if only one component was present. This low value may perhaps depend upon an accident. A lysin seems not to dissolve the blood corpuscles, which are combined with another lysin.

The hæmolytic action of bases is in a very perceptible manner decreased, when *normal serum* is added. The action of various kinds of serum seems to differ. Two different kinds have been used, one marked "égoutteur", the other was prepared just before the experiments. The former serum is in the table designated A, the latter B. The experiments are carried out with solutions which are $^1/_{40}$ n in the case of sodium hydrate

Amount cc.	$^1/_{40}$ n NaOH	A III	A II	A I	B III	B II	B I
1.0	100*	60	60	47	60	27	7
0.8	75	53	40	37	40	22	4
0.6	41	40	18	25	20	15	2
0.5	27	22	15	22	12	4	1
0.4	7	7	7	10	5	1	0.7
0.3	1	„	„	„	„	„	„
	$^1/_{40}$ n NH_3	A III	A II	A I			
1.0	60	45	40	33			
0.8	45	40	33	30			
0.6	22	27	27	27			
0.5	13	12	15	16			
0.4	4	5	5	„			
0.3	1	„	„	„			

[illegible]

or ammonia. The most concentrated solutions (I) contain 87,5 $^0/_0$ serum, the following (II) 37,5 $^0/_0$ and the weakest (III) 15 $^0/_0$. Much weaker solutions have been examined but they had no effect.

It appears that the action of serum B is much stronger than that of serum A; and the action upon sodium hydrate is stronger than the action upon ammonia. In the case of solution A III, the decrease is very insignificant, as regards ammonia hardly recognisable, and as regards sodium hydrate about 10 $^0/_0$ (when the greatest amounts are added). For the solution A II, the decrease in the strongest concentrations amounts to 25 $^0/_0$ for NaOH, to 15 $^0/_0$ for NH_3, in the weaker (0,5 cc.) to 10 $^0/_0$. For NH_3 it is not perceptible. The strongest solutions of A I decrease by about 40 $^0/_0$ for NaOH and 25 $^0/_0$ for NH_3. The strongest solutions of B III decrease by about 25 $^0/_0$, the strongest of B II by 50 $^0/_0$, and the strongest of B I up to 60 $^0/_0$. The strongest solutions correspond after having been mixed with blood to 1,4, 3,4 and 8 $^0/_0$ of the whole fluid. The action is thus comparatively slight.

This is, in a still higher degree, the case with *egg albumen*. For the following experiments, as for the earlier ones, $^1/_{40}$ n solutions of the bases have been

Amount cc.	NaOH	Na II	Na I	NH_3	NH_3 II	NH_3 I
1.2	„	47	47	„	40	40
1.0	50	43	40	33	37	33
0.8	40	35	33	30	30	29
0.6	23	25	22	15	20	20
0.5	10	12	15	7	10	15
0.4	3	4	„	3	3	5
0.3	2	„	2	„	„	„

used, to which 15 resp. 33 °/$_0$ fresh egg albumen have been added. As usually, a 2,5 °/$_0$ blood dilution in NaCl was used. Solutions of egg albumen, more diluted than those just mentioned, have no recognisable effect. The stronger solutions are signified by I and the weaker by II.

The egg albumen has no recognisable effect upon ammonia; the action upon sodium hydrate is, in the case of solution I, about 20 °/$_0$, in the case of solution II still smaller, this applies to the highest concentrations. It seems from the figures as if the egg albumen in the lowest concentrations increased the hæmolytic action a little. This depends probably on the slower sedimentation of the blood-corpuscles in the solutions containing albumen. It is therefore very likely that if this perturbing circumstance did not exist, the lowering action of the egg albumen would be a little greater than the experiment shows.

Probably the action of albumen is of an exactly similar nature to that of normal serum. It is a well know fact that the albumens have the property of combining with both acids and bases. It is without doubt this property of the albumen which concerns us here. Ammonia, as a comparatively weak base, is in this dilution not able to combine with the albumen in a recognisable degree, its combinations being almost completely hydrolysed in such great dilutions.

Normal sera and especially serum B have a greater power of combining than egg albumen. This may be attributed to, first that they may be considered as stronger acids than egg albumen, and still more probably that each cc. of the serum contains more valencies, capable of fixing bases, than 1 cc. of the egg albumen. It may be noted, in this context that serum B was rather putrified.

III

The albumens have a much greater power of decreasing the hæmolytic action of the tetanolysin than of the hæmolytic action of bases. As regards the normal serum, this is already well known, and its action has been compared with that of antitoxin, although it is much more complicated, as will be seen later.

To begin with, we give below the results of experiments with egg albumen. First, the amount of added toxin is indicated as in the earlier tables. In the first series it consisted of 0,2 $^0/_0$ lysin, in the last (sixth) — half of 2 $^0/_0$ lysin and the other half of egg albumen, in the fifth the amount of lysin was 1,5 cc., the amount of albumen 1 cc., to which 1,5 cc. of water was added; in the fourth series the corresponding amounts were 1,5, 0,5 and 2 cc.; in the third 1, 0,2 and 2,8 and in the second 1, 0,05 and 2,95.

For the sake of clearness the amount of lysin is indicated in the terms of 1 : 100 $^0/_0$ of the whole mixture to which blood diluted with salt solution was used; these are given in the table in brackets by the side of the figures expressing the amount of hæmolysis.

Amount cc.	Lysin	1.25 A	5 A	12.5 A	25 A	50 A
1.0	30 (1.8)	100*(4.5)	47 (4.5)	33 (6.7)	43 (6.7)	40 (9.0)
0.8	27 (1.47)	100*(3.7)	33 (3.7)	29 (5.5)	37 (5.5)	35 (7.4)
0.6	13 (1.12)	„	30 (2.8)	27 (4.2)	33 (4.2)	32 (5.6)
0.5	12 (0.95)	„	18 (2.4)	18 (3.6)	25 (3.6)	30 (4.7)
0.4	9 (0.77)	35 (2.0)	17 (2.0)	15 (3.8)	22 (3.0)	„ (3.9)
0.3	„	27 (1.5)	„	„	„	„

The last of the values for 1,25 A shows that egg albumen has no sensible action in a concentration of 0,04 $^0/_0$. The middle figures in the next column indicate that already 0,3 % egg albumen is able to decrease the action of the lysin in the proportion 2,8 : 1,8, viz. by about 35 %. The corresponding ciphers in the next column show that 0,75 % albumen decreases the action of the lysin to about 64 %. The double amount of albumen has almost the same power of action. The experiments showed this action to be smaller, which without doubt arose from errors of experiment. — And 2,5 % albumen decreases in the proportion 4,7 : 1,8, viz. by 62 %.

The facts here brought forwards are quite characteristic. The action of albumen is not perceptible in the concentration 0,04 %, it then increases rapidly with the concentration, so that it, when this is 0,3 % amounts to 35 %, and when it is 0,75 % to 64 %, and after that it remains nearly constant. When the percentage of albumen rises from 0,3 to 0,75 the increase in the action is almost proportionate to the amount of albumen (with allowance for errors of experiment).

The way in which the figures follow each other in these series is also very remarkable. In the first series, in which pure tetanolysin was added, the values of hæmolysis (apart from the first) follow approximately the rule that hæmolysis is in proportion to the square of the amount of lysin added. But in the fourth series (12.5 A) on the other hand, proportionality is nearly maintained betwen the amount of toxin and hæmolysis, and in the last two series the increase of hæmolysis is less than proportionate to the amount of toxin.

One might be tempted to suppose, that this arose from the greatest amounts of albumen accompanying the greatest amounts of toxin in the experiments. But we have seen above, that beyond a certain limit which has been exceeded in this case (series 50 A), the increased additions of albumen have no effect. Albumen and serum have in this repect the same effect upon sodium hydrate and ammonia, i.e. the action upon the higher concentrations of the hæmolysing body is proportionately much greater than upon the lower concentrations. It would therefore seem as if the albumens added diminish the strength of the chemical combination, which the hæmolysing bodies, to begin with, form with the red blood corpuscles. We will return to this phenomenon later on.

The normal serum has, as above mentioned, a very great effect upon the tetanolysin. In the table below, the figures indicate the amount of serum added to the toxic fluid, multiplied by 100000.

Amount cc.	0 S.	13 S.	27 S.	81 S.	310 S	870 S.	1250 S.	2500 S.	6250 S.	50000 S.
2.0	„	„	„	„	„	„	„	„	2	2
1.6	„	„	„	„	„	„	„	22	1	1
1.3	„	„	„	„	„	„	„	17	1	1
1.0	„	„	„	„	„	65	53	15	0.5	1
0.7	„	„	„	„	67	65	47	13	„	„
0.5	„	„	„	„	67	60	29	10	„	„
0.4	„	„	„	45	„	„	„	„	„	„
0.35	„	„	„	„	40	40	27	5	„	„
0.3	„	„	36	33	32	„	„	„	„	„
0.25	„	„	30	24	27	30	27	3	„	„
0.2	„	30	25	15	18	20	18	2	„	„
0.15	26	21	15	12	10	12	10	2	„	„
0.1	14	10	7	5	„	7	7	„	„	„
0.07	7	7	4.5	3	„	„	„	„	„	„
0.05	4	4.5	2	„	„	„	„	„	„	„
0.04	3	3	„	„	„	„	„	„	„	„
0.03	2	1.5	„	„	„	„	„	„	„	„
0.025	1.5	„	„	„	„	„	„	„	„	„

As usual a 2,5 $^0/_0$ blood dilution in salt water was used. The figures before S indicate how many 100 000 parts of serum were contained in the fluid added to the blood. Already, when the serum reaches an amount of 1,3 million parts of the bloodmixture, (addition of 0,1 cc. 0,013 $^0/_0$ toxinfluid to 10 cc. blood emulsion) its action is perceptible. The action increases nearly in proportion to the amount of serum, until this has reached 16 million parts (= 0,2 cc. of 0,081 $^0/_0$). In this case the decrease in the action of lysin is about 50 $^0/_0$. During the increase of serum from 16 to 250 million parts, the action remains just the same. But from 250 million parts the action of the serum becomes again effective.

The figures in the last column are very unreliable, because the blood dilution becomes strongly coloured (yellow), when greater amounts of serum (5—10 $^0/_0$) are added, and by this means the hæmolysis appears much greater than it really is. And the determinations are generally rather unreliable, when the hæmolysis is as slight as here (under 2). It is also observed in this case that the increase of hæmolysis with increasing amounts of toxin becomes slower as more and more serum is added.

It appears from this circumstance how necessary it is in these experiments to wash away the normal serum from the blood corpuscles, before they are used. The following experiment was carried out, partly with blood corpuscles, twice washed with an isotonic NaCl solution and centrifugalised, partly with corpuscles from the same blood, only once centrifugalised and not washed. The difference is very evident (amounting to about 30 $^0/_0$). The lysin was a 0,1 $^0/_0$ solution, the blood dilution 2,5 $^0/_0$.

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One must use about 50 % more lysin in order to have the same effect on the "unwashed" blood. This quantity may evidently be different in different cases.

For our experiments we therefore only used blood corpuscles which had been twice very thoroughly washed.

As the albumen or serum added to ammonia or sodium hydrate has a very different effect from what it has when added to tetanolysin, we cannot assume that this action concerns the blood corpuscles; in that case the effect ought to be the same in both cases. We must therefore suppose that the serum or albumen has a direct action upon the lysin or some combination of it. As we hawe seen, the tetanolysin probably combines with red blood corpuscles, and under such circumstances it is very likely that also the albumen or serum or perhaps some of their components combine with tetanolysin, which in this way becomes weakened. The amount of tetanolysin combination formed is nearly proportionate to the added amount of albumen or serum, but only to a certain point, at which practically all tetanolysin has been transformed to the weaker com-

Amount added cc.	Bloodcorpuscles	
	washed	unwashed
1.5	27	12
1.0	14	7
0.7	7	4
0.5	4	3
0.4	3	1.5
0.3	2.5	1
0.25	1.5	0.6

bined form. From this point further additions of albumen have no appreciable effect.

Such is on the contrary not the case for the normal serum. When a gradually increasing quantity of this is added, the hæmolytic power of the tetanolysin is decreased. It is curious that in the case of such a strong dose as 1250 S this property is not more conspicous than in the above table. This most probably depends on an accident, as appears from the following experiment which is carried out with 2,5 $^0/_0$ blood, diluted with 7,8 $^0/_0$ cane sugar solution.

Amount cc.	0 S	13 S	310 S	620 S	1250 S	2500 S	6250 S	50000 S
1.0	"	"	"	13	8	4	3.5	2
0.7	"	"	14	11	4	4	"	"
0.5	"	16	10	9	4	3.5	"	"
0.4	"	6	6	7	3 5	3.5	"	"
0.2	15	5	3	3	3	"	"	"
0.1	6	3	"	"	"	"	"	"
0.06	4	"	"	"	"	"	"	"

In this case the second effect of serum is more distinct than in the preceding case and is perceptible already with 620 S.

On the other hand the former table is more instructive as regards the action of the smallest doses of serum. After this is appears that *normal serum in higher doses has a real neutralising action upon tetanolysin.* A body with such an effect, viz. antitetanolysin will be examined below. *The normal serum may be considered as composed of a small amount of antitoxin and great amounts of albumen.*

Additions of proteids, normal serum included, have thus a decreasing action upon hæmolysis. They behave as if they combined partially with the bases and with the tetanolysin, giving in this way weaker hæmolysing agents. Therefore it is characteristic of all these proteid bound hæmolytic bodies that the increase of hæmolysis with increasing amounts of the hæmolysing body is progressing less rapidly than for the pure bases or lysin. This effect increases — as may be expected — with the amount of proteid.

In this chapter we have dealt with the *action of a few so to speak neutral bodies upon the hæmolysis.* This action is very different in different cases and must therefore be ascribed to different causes.

As to the action of *neutral salts upon the strong bases,* it is similar in all of them (when present in equivalent amounts). This is most easily explained by supposing that the combination of strong alkalies with red blood corpuscles behaves as weakly dissociated salt like acetate of copper or potassiumstibiotartrate or like a rather strong base, the degree of dissociation of which is decreased through the addition of salt.

The same applies to the effect of *ammonia salts upon the hæmolytic action of ammonia,* but the effect is here much stronger. In other terms, the combination of ammonia with blood corpuscles is much less dissociated than the combinations of the strong bases. It is only the dissociated molecules of these salt like combinations which react (or at any rate they react much quicker than the undissociated). Therefore the velocity of reaction is decreased through the addition of salts, containing the same ions. We might also suppose that some soluble product of the combination of the bases with the red blood corpuscles was formed and that this was necessary for the further progress of the reaction. The solubility of this body, which would be an electrolyte, would be decreased through addition of similar ions. In this case it must also be supposed that the solubility or the degree of dissociation of the salts of the strong bases (KOH, NaOH and LiOH) were almost similar, while on the contrary the solubility (or the degree of dissociation) of the salt of ammonia is considerably smaller. The small effect of the alkaline earths probably depends on the formation of insoluble products of the hæmolysis, which therefore in no higher degree can manifest itself.

But the *salts of the strong bases and of ammonia* have also another effect upon hæmolysing processes having an increasing effect upon tetanolysin. This probably depends on a direct action on the red blood corpuscles, which through the addition of salt are rendered, so to speak, more susceptible of attack. This effect is of course supraposed to that just mentioned, which in consequence is apparently weakened. Still the increasing effect of salts upon hæmolysis can be demonstrated by the examination of the velocity of reaction. 1 cc. n NaCl solution added to a solution of 0,5 cc. 0,5 n NaOH in 10 cc. 2,5 % blood emulsion in cane-sugar solution, at 22,4° C increased the velocity of hæmolysis by about 25 %; when half the amount of NaCl was added, the velocity was increased by half that quantity. Sodium nitrate seems in these experiments to have about the same action as NaCl, perhaps a little stronger; the action of the sodium sulphate on the contrary is only half as strong. All the ammonia salts have, on the other hand, a strong decreasing effect on the velocity of reaction, yet not to such a great extent as in the above experiments. The sulphate, the chloride and the nitrate had in this case almost the same effect: when 1 cc. normal solution was added to a mixture of 0,5 cc. 0,2 n ammonia with 10 cc. blood (2,5 $^0/_0$ in cane-sugar solution) the velocity of reaction was decreased to about $^1/_3$, and by the addition of 0,25 cc. normal solution it was only $^6/_{10}$ of the velocity it reached, when salts were absent. In this case the increasing effect of the salts was not able to outway the decreasing effect of ammonia salts upon the degree of dissociation of the combination of ammonia with red blood corpuscles.

The *proteids* have quite another effect. This appears most plainly in the action of egg-albumen upon tetanolysin. We have seen that this action is, roughly speaking, proportionate to the proteids added, up to a certain point, and then becomes almost stationary, in spite of further additions of proteids; it is therefore probable that in this case a rather strong combination takes place. These experiments suggest, that a combination is formed between tetanolysin and proteid, which retains the properties of tetanolysin, although to a less extent. It follows that the hæmolytic effect of the lysin is decreased, this being especially the case with higher concentrations.

The *normal serum* has a precisely similar effect upon tetanolysin. But to this effect is added another property of the normal serum, which is evident in higher concentrations and seems to correspond to the action of antitoxin upon toxin. As will be shown in the following, this process must be considered as probably a phenomenon of neutralisation.

According to this, the action of albumen and serum upon sodium hydrate and ammonia may be explained as follows: sodium hydrate or ammonia is added to albumen or serum, by this means combinations are formed which in the main keep the properties of both bases, but are weaker. Also another explanation could be given, viz. the proteids act partly as acids and neutralise the bases, resulting in the formation of saltlike combinations which still were highly hydrolysed, especially in the case of ammonia. These interpretations do not after all differ very much from each other and lead in the main to the same conclusions.

THE ACTION OF ANTITOXIN UPON TOXIN.

Numerous investigations concerning this problem have shown, that the toxicity of a toxin decreases when increasing amounts of antitoxin are added. The first experiments of this kind with diphtheria toxin and with tetanolysin were represented as a so called "toxin spectrum", which in the case of tetanolysin had the form, shown in fig. 3, but it was already then pointed out, that the action of antitetanolysin upon tetanolysin should rather be expressed by means of a continuous curve.[1])

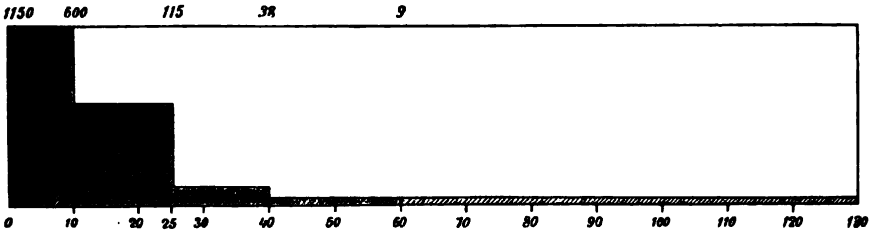

Fig. 3.

During the last three years a very great number of experiments with tetanolysin were undertaken, and at last one was obtained which showed very regular results. The whole experiment was carried out on one day with the same blood-dilution and all the values therefore are completely commensurable. They are given in the following table, where the first column indicates how many (n) cc. of antitoxin-solution ($^1/_{400}$ %) were added to 2 cc. of a 2 % tetanolysinsolution and to (2 — n) cc. water. Under the heading „x obs.“ is indicated the "toxic quantity" i. e. the quantity of this mixture, which added to 10 cc. 2,5 % horseblood (diluted with saltwater) produced a hæmolysis = 20. When amounts over 1 cc. are indicated, only $^1/_{10}$ of a 10 times more concentrated solution was added. These cases are marked by an asterisk in the table below. Under the supposition that all the tubes, with the same degree of hæmolysis, contained the same amount of toxin, the amount of toxin which was present in every cc. of the solution added was calculated in arbitrary units. In reality, we calculated how much toxin was present in the whole mixture of 10 cc. blood and the added amount of toxin (this amount of toxin being proportionate to the total volume); and it was then supposed that if this fluid, the blood corpuscles excepted, was concentrated to the original volume of toxin added, this ought not to cause any change in the toxicity — or if a such should really be present, that it would be of the same relative size in all cases. The observations below mentioned show this supposition to be in concordance with the experience. Is x the toxic quantity, the toxicity G will be found after the following formula:

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[1]) Th. Madsen l. c. p. 226.

$$G = \frac{1}{x} \cdot \frac{10 + x}{10}$$

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Should the volume added be only one tenth, then 0,1 x is substituted for x in the numerator, the x in the denumerator remains unchanged.

Besides x obs. is a column x calc., calculated in the manner indicated below. In the next column under the heading G obs. is indicated the G, calculated from the x obs. as above mentioned. The last column, G calc., gives a theoretical value, calculated after a formula indicated below. From this G calc. a x calc. has been found according to the above formula for the purpose of a direct comparison with the observed value of x.

The toxicity of mixtures of tetanolysin and antitetanolysin.

n	x obs.	x calc.	G obs.	G calc.
0	0.23	0.23	4.45	(4.45)
0.05	0.28	0.28	3.67	3.67
0.1	0.33	0.35	3.13	2.95
0.15	0.45	0.46	2.32	2.29
0.20	0.66	0.60	1.62	1.76
0.30	*1.15	1.05	0.97	1.03
0.40	*1.6	1.64	0.63	0.62
0.50	*2.3	2.21	0.45	0.46
0.70	*3.8	3.7	0.27	0.28
1.0	*>6.0	5.9	0.18	0.18
1.3	*9	8.0	0.12	0.13
1.6	*12.5	10.2	0.09	0.11
2.0	*14	13.0	0.08	0.09

These observations are graphically reproduced in fig. 4, where the line represents x calc. and the dotted line G calc. The observed values are indicated by means of this cross (×) or this (+) cross.

It appears that the values of x obs. increase continuously and gradually with n. In consequence the

G obs. continually decrease with increasing n. There is therefore no reason for drawing the G curve (the previously so called "toxinspectrum") as a line, formed like stairs, this would also be very improbable from a theoretical point of view. It was owing to the great errors of experiment and especially the very complicated composition of a deteriorated diphtheria toxin which caused the first curves of toxicity to take this form.

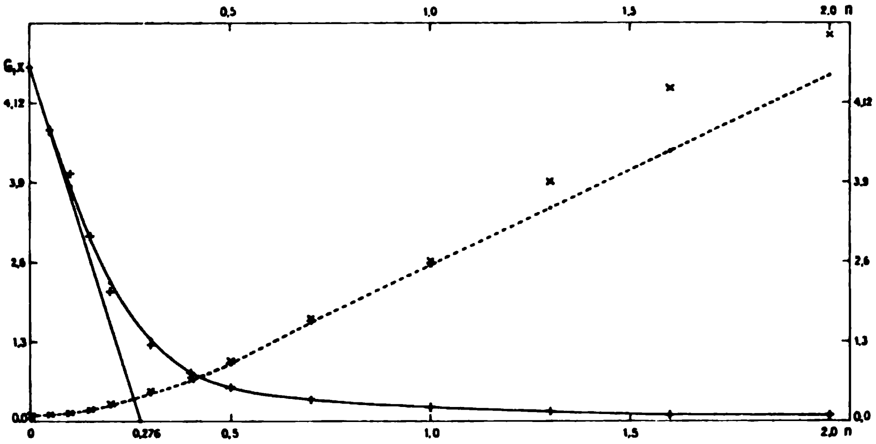

Fig. 4.

Neutralisation of tetanolysin by antitoxin

+ × observed values.

— toxic quantity
- - - toxicity

This curve of toxicity is very like that, which represents the equilibrium between a body in partial dissociation and its products of dissociation. The formula

$$\left\{\frac{\text{Free toxin}}{\text{vol}}\right\}\left\{\frac{\text{Free antitoxin}}{\text{vol}}\right\}=K\left\{\frac{\text{combined toxin-antitoxin}}{\text{vol}}\right\}^2$$

has been used for the calculation of G. The calculation of the free and combined toxin has been made in the following manner. The original mixture of toxin and blood (free from antitoxin) contains pr. cc. 0,23: 10,23 units (as unit is chosen the amount of toxin, contained in 1 cc. of a 1 % solution of tetanolysin). When, in another experiment, x cc. must be added to obtain the same degree of colour, then the amount of toxin pr. cc. is x : (10 + x).

As in both experiments the same degree of colour resulted, the amount of free toxin must be the same in the two cases (a correction for the variations of the amount of blood is not necessary, cfr. p. 15) — viz 0,23 : 10,23, the amount of toxin combined is consequently the difference between the added and the free toxin = x : (10 + x) — 0,23 : 10,23. The amount of anti toxin combined is the same. As we now have added n cc. antitoxin solution to 2 cc. 2 % lysin, $\frac{n}{4}$ cc. antitoxin corresponds to every unity of toxin added. Then in each cc. of solution $\frac{n}{4} \cdot \frac{x}{10+x} \cdot p$ parts of antitoxin are present supposed that 1 cc. of the used solution of antitoxin is equivalent to p cc. of 1 % tetanolysin. From this amount the amount of antitoxin just now determined is subtracted, and then the value of the amount of free antitoxin is found. Thus we get the equation:

$$\left(\frac{0,23}{10,23}\right)\left\{\frac{n}{4}\frac{x}{10+x}p-\left(\frac{x}{10+x}-\frac{0,23}{10,23}\right)\right\}=K\left(\frac{x}{10+x}-\frac{0,23}{10,23}\right)^2$$

For the determination of the values of p and K, representing the two unknown, as many equations (12) can be set up as we have experiments concerning the toxicity of mixtures of toxin and antitoxin. By means of successive approximation first the most probable value of p is found, and after that the most probable value of K. The values calculated from the above data as the most probable are: $\frac{1}{p} = 0{,}069$; $p = 14{,}55$ $K = 0{,}115$.

III

$p = 14{,}55$ indicates, that 1 cc. of the antitoxin used $^1/_{400}$ ($^0/_0$) is equivalent to 14,55 cc. of the above supposed arbitrary unit of toxin. Or in other terms: the unit of toxin used corresponds to 0,069 cc. of the antitoxin used.

In Fig. 4 this circumstance is represented by the fact that the tangent of the G-curve in the point $n = 0$ cuts the abscissa in the point $n = 0{,}276$, for 0,276 cc. of $^1/_{400}$ $^0/_0$ antitoxin are sufficient to neutralise 2 cc. of 2 $^0/_0$ lysin i.e. 4 toxin-units that are used in making the toxic mixture.

By means of the values of p and K later on the values G calc. and x calc. have been calculated. The concordance between the observed and calculated values of x is exceedingly good. The lacking concordance, which perhaps might be found in the case of the highest values of n, for inst. $x_{obs.} = 12{,}5$; $x_{calc.} = 10{,}2$ is more apparent than real, for in the case of these great amounts of antitoxin the equilibrium between fixed and free antitoxin and toxin is obtained very slowly. It is therefore necessary to permit the solutions of toxin and antitoxin to be mixed for a long time, before they are used for the experiments. But the tetanolysin is weakened, when it is kept in solution during a longer time, and any special good concordance can therefore not be expected in this case. All experiments with great amounts af antitoxin (from and with $n = 0{,}50$) give also a too great value of $x_{obs.}$ compared with $x_{calc.}$ In other terms: the toxin was weaker than would be expected from the experiments with small amounts of antitoxin. The toxin becomes weaker through the long time of reaction, and as a rule the more so the longer time the mixture of toxin and antitoxin has to be kept before it is used.

III

Besides p and K also another constant, or to put it more exactly, two constants, viz. two of the powers in the above equation, must be determined by means of the material. Yet in this case only simple integers can be used, so that the choice is not difficult, a hasty glance on the figure giving the most probable values. It must still be noticed, that the powers 1 and 2 represent as simple a case, as could be expected, and this makes it more probable that the equation represents a natural process.

For the purpose of illustrating still more thoroughly the great resemblance between the neutralisation of toxin through antitoxin and of a base through an acid, we have examined a closely allied phenomenon, where a base ammonia, also acting as a lysin, is neutralised by an acid the boracic acid. This case could be examined with quite the same method which was used with the combination of toxin with antitoxin. Here we know beforehand p, as the amount of boracic acid, equivalent to a certain amount of ammonia or, to put it more exactly, very few values of p were left to choose between. We might suppose either that one molecule of ammonia NH_3 combined with one molecule boracic acid H_3O_3B to $NH_4H_2O_3B$, or that two molecules H_3N combined with 4 molecules boracic acid to the salt $(NH_4)_2\ B_4o_7$ (+ 12 H_2O), analogous to borax. Also other possibilities might be supposed, viz. that two or three molecules of ammonia combined with one molecule boracic acid. The experiments showed the first supposition — the simpler one — to be right. Solutions were made, which to 1 molecule of ammonia contained 0, $^1/_6$, $^1/_3$, $^2/_3$, $^3/_3$, $^4/_3$, $^5/_3$ and 2 molecules of boracic acid. Of these mixtures various doses were added to 10 cc. blood emulsion (2,5 $^0/_0$ in saltwater) and put at 37° C. for 1 hour, quite as the mixtures of toxin and antitoxin in the example just mentioned. Among the mixtures of ammonia, one was selected, containing 0,17 cc. 0,1 n NH_3 in 10 cc. blood, which showed a convenient degree of colour that could be exactly determined by means of the colorimetric method.

In this case theoretical reasons lead us to foresee that the equation:

$$\left\{\frac{\text{free } NH_3}{\text{vol}}\right\}\left\{\frac{\text{free } H_3O_3B}{\text{vol}}\right\} = K\left\{\frac{NH_4H_2O_3B}{\text{vol}}\right\}^2$$

would be right.

If (NH_3) be the concentration of ammonia, (H_2O) be the vapour-pressure of the water in the solution, which, in these exceedingly small concentrations without recognizable error, can be considered as constant (the total concentration amounts in maximo to 0,025 normal, corresponding to a decrease of the vapour-pressure of only $^1/_{22}$ percent) — is (H_3O_3B) the concentration of the boracic acid etc., then the following equations are valid:

$$(H_3N)\ (H_2O) = K\ (NH_4OH) = K_1\ (NH_4)\ (OH)$$
$$(H_3O_3B) = K_2\ (H)\ (H_2O_3B)$$

from this follows:

$$(H_3N)\ (H_3O_3B) = K_3\ (NH_4)\ (H_2O_3B)$$

it is: the product of the concentration of the undissociated ammonia (not combined as salt) with the concentration of the free boracic acid is proportionate to the product of the concentrations of the ions of NH_4 and H_2O_3B. If the amount of boracic acid be not too small for a recognizable amount of borate to be formed, then the quantity of NH_4 — and H_2O_3B ions, which may not be considered as arising from dissociated salt[1]), but from dissociated ammonia and boracic acid, is minimal in comparison with those ions, arising from dissociated salt. In the present case the blood is emulsionated in 0,8 $^0/_0$ Nacl (= 0,14 n). The quantity of salt, formed through combination with ammonia, never amounting to 0,006 normal in the above experiments, the total concentration of the different solutions may be considered as subjected to only such small variations that the degree of dissociation of the salts keeps almost completely constant. Therefore the number of ammonium ions is proportionate to the amount of ammonia combined, and the number of borateions proportionate to the amount of boracic acid combined, which is equivalent to the amount of ammonia combined.

Thus we get the following equation:

$$(\text{amount of free } NH_3)\,(\text{amount of free } H_3O_3B) = K\,(\text{amount of combined } NH_3)^2$$

This is exactly the same equation as previously found in the case of tetanolysin, when toxin is exchanged for ammonia and antitoxin for boracic acid. In this case the experiment is not so simple as in the case of lysin, the newly formed ammonia salt decreasing the action of ammonia. Still with these small amounts it can be concluded without recognizable error, that when ammonia-salt is added in an arithmetical progression, the hæmolysing property of ammonia decreases in geometrical progression. In the following calculation our starting point was the fact, previously ascertained, that 0.01 n ammoniumsalt decreases the action of ammonia to 40 percent. If thus the degree of hæmolysis used as unit is produced by 0,00167 n ammonia, as above stated, then in the presence of 0.01 n borate of ammonia 0,00417 n free ammonia is needed, and in the presence of 0.005 n borate of ammonia 0,00264 n ammonia is needed to produce the same degree of hæmolysis. All the cases are chosen in such a way, that they produce a similar degree of hæmolysis, in consequence the calculations of the amount of free ammonia are undertaken in the above manner.

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[1]) Cf. S. Arrhenius: Zeitschr. f. physic. Chemie. 5 1 (1890).

In the following table *n* indicates the amount of boracic acid (in equivalents) present in the solution for each equivalent of ammonia. The ammonia used for the mixtures was 0.1 n, the solution of boric acid 0.2 n and they were mixed in different proportions without water being added. x obs. indicates the amount of the mixture in question, which added to 10 cc. of blood produced the hæmolysis intended. In the next column a calculated value, x calc., is given. Under the heading *a* the amount of ammonia (free or combined), present in every test tube is indicated in 0.01 normal solution as unit. Under *b* the corresponding amount of boracic acid added is indicated. Under *c* obs. is found the amount of free ammonia, which in the present case is

The toxicity of mixtures of NH_3 and H_3O_3B.

n	X obs.	X calc.	*a*	*b*	c obs.	c calc.	G obs.	G calc.
0	0.17	(0.17)	0.167	„	0.167	(0.167)	6.0	6.0
1/6	0.20	0.22	0.181	0.030	0.169	0.154	5.1	4.7
2/6	0.25	0.27	0.209	0.070	0.172	0.157	4.1	3.8
4/6	0.40	0.42	0.289	0.193	0.184	0.174	2.6	2.5
6/6	0.70	0.66	0.436	0.436	0.207	0.219	1.51	1.62
8/6	0.90	0.99	0.495	0.660	0.216	0.213	1.21	1.10
10/6	1.50	1.43	0.712	1.187	0.256	0.268	0.77	0.79
12/6	2.00	2.00	0.833	1.667	0.279	0.279	0.60	0.60

necessary for the production of the given hæmolysis, calculated according to the above reasons. c calc. is calculated from the above equilibrium-formula, in which the constant K is put = 1.02. Finally the table under the headings $G_{obs.}$ and $G_{calc.}$ contains two columns, indicating the observed and the calculated toxicity of the mixtures in question in the same manner as in the case of tetanolysin.

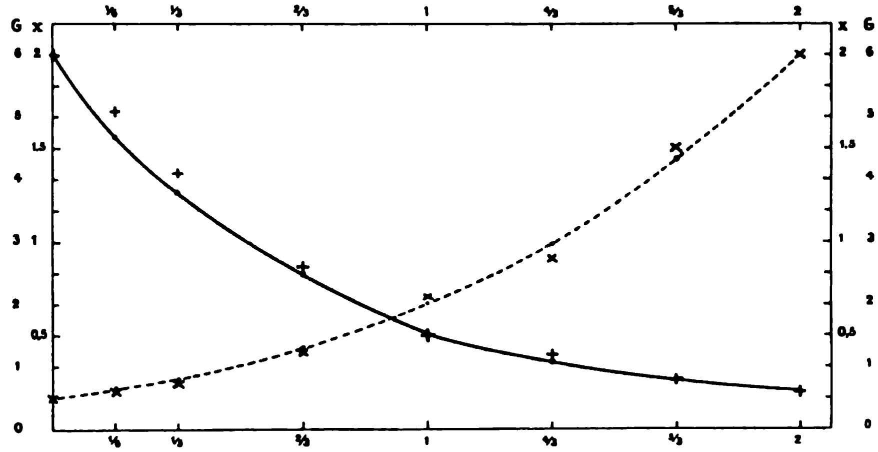

Fig. 5.

Neutralisation of ammonia by boracic acid.

+ × observed values.

— toxic quantity

- - - toxicity

The concordance between the observed and the calculated values is quite satisfactory and the difference not greater, than may be explained by errors of experiment. The experiments 2—4 have throughout given lower values of $c_{calc.}$ than of $c_{obs.}$ In this case the dissociated parts of ammonia and boracic acid play perhaps a perceptible role, and it is worthy of notice, that a correction in this direction would increase the agreement, but we want observations at a temperature of 37° C., by means of which this correction could be made.

The curves of fig. 5 are made analogous to that of tetanolysin (fig. 4); an out drawn line representing the values of a, with upright standing crosses indicating the corresponding observed values, and a dotted line the calculated and observed values for $1 : a$. The latter corresponds to the curve of toxicity and ought according to the old nomenclature to be called the toxin spectrum of ammonia. There is a striking agreement between this curve and the corresponding curve for tetanolysin. They only differ as regards the constants, these being different in the two cases (0,117 for tetanolysin, 1,02 for ammonia). How insignificant this difference is appears from the fact, that tetanolysin, kept in solution for a certain time, becomes weaker; this effects an increase of the constant. As will later on be shown, this constant during the time May 1900—June 1902 had increased from 0.12 to 1.7 and had thus once passed the value 1.02. If the toxin had been examined at this time, the curves of toxicity of ammonia and tetanolysin had been identical.

The consequence of all this is, that the decrease of the action of tetanolysin through antitoxin in all likelyhood is caused by a combination of the two bodies. As so often occurs, especially in the case of organic combinations, this combination is partly decomposed in its components, so that a chemical equilibrium between the latter and the combination takes place according to the above formula (the law of Guldberg-Waage). This formula indicates that of one molecule of toxin and of one molecule of antitoxin two molecules of the combination toxin-antitoxin are formed.

The great likeness between the state of equilibrium between ammonia, boracic acid and ammonia borate and their ions, needs not go so far that it is necessary to suppose, that also the toxin-antitoxin is electrolytically dissociated, and that it has the constitution of a salt; and as little the lysin needs to be considered a base and the antitoxin an acid. The experiments made in this direction, certainly not very systematic, indicate that matters do not stand so, but that here rather a case of common dissociation is present. Perhaps it is also worth notice, that according to the above equation the dissociation of the toxin-antitoxin is not changed with the dilution.

The resemblance between the combination of toxin through antitoxin and the neutralisation of a base by means of an acid was already early searched for. The non success in detecting this resemblance depends on, that the neutralisation of a base through an acid was always considered to take place as in the case of strong bases and strong acids, where complete neutralisation occurs, when they are mixed together in equivalent amounts. This phenomenon is represented by the straight line on fig. 6.

If a strong acid, for instance hydrochloric acid, be added to a not all to weak base (f. inst. ammonia), the action of NH_3 as base (f. inst. its hæmolytic action) is completely suppressed, a soon as an equivalent amount of acid is added. If only half an equivalent of acid be added to one equivalent of the base, then its action is reduced to the half and the addition of one third equivalent of acid will reduce the action of the base to $^2/_3$ and so on. The neutralising action of the acid is proportionate to the amount added until complete neutralisation takes place; after this the acid has no more neutralising action. But the difference between this phenomenon and the neutralisation of, f. inst., ammonia with boracic acid or lysin with antilysin is in reality only of a qualitative nature. To both cases applies the equation:

$$\text{(Amount of free acid) (Amount of free base)} = \text{K (Amount of combined base)}^2.$$

In the case of the strong acids and the strong bases (or even NH_3) K is = 0, perhaps more correctly exceedingly small. It follows from this that either the amount of free acid or the amount of free base is exceedingly near 0, viz. a strong acid and a strong base cannot exist in perceptible amounts in the same solution. It is quite another matter when K has a definite value, f. inst. 1, as it is closely the case, when boracic acid combines with ammonia. If (n—1) equivalents of acid be added to 1 equivalent of ammonia, then 1/n of ammonia will always remain free, because:

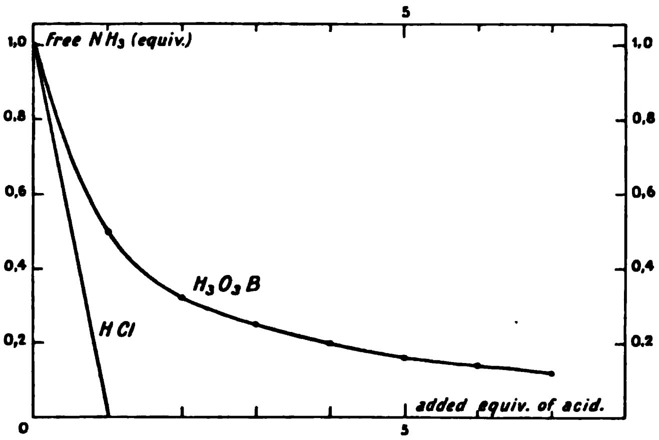

Fig. 6.

$$\frac{1}{n}\left\{(n-1) - (1-\frac{1}{n})\right\} = (1-\frac{1}{n})^2$$

It appears that if one equivalent of boracic acid is added, then the half of the base is free, if two equivalents be added one third is free, and if ten equivalents are added then yet one eleventh part of the base is free. The curve representing this phenomenon, the upper curve in fig. 6 is tangent to the upper end of the line, which represents the course of neutralisation of a strong acid — and on the other hand for higher additions of acid it approaches to an equilateral hyperbola ($y = \frac{1}{x}$).

As before said this last mentioned curve gives an almost exact representation of the neutralisation of ammonia with boracic acid (at 37° C in a solution not too concentrated). If the action of ammonia as hæmolysin had been investigated, then ex analogia a similar spectrum as in the case of usual toxin or tetanolysin (fig. 3) had been made and the following conclusion had been drawn: Boracic acid (antitoxin), added in the amount 1 to ammonia neutralises 50 $^0/_0$ of this base; added in the amount of 2 neutralises 66,7 $^0/_0$, in the amount of 3 75 $^0/_0$ and in the amount of 4 80 $^0/_0$; from this follows, that whereas for each time the respective amounts of toxin 50, 16,7, 8,3 and 5 $^0/_0$ are neutralised by the same amount of boracic acid, the first amount of ammonia neutralised is three times as toxic as the amount neutralised next, which again is double as toxic as the next amount; the latter is $1^1/_2$ times more toxic than the following etc. In other terms ammonia is no simple body, but consists of several components of different toxicity (and these toxicities are in simple reciprocal proportions). Of these components the toxin with the greatest chemical affinity is first neutralised[1]).

A similar conclusion has been drawn in the case of toxin, and the toxin first neutralised (the strongest) has been called proto-toxin, the next deutero-toxin, the next trito-toxin etc. The last very weak toxins were called toxons.

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[1]) From considerations, which can hardly be maintained, Danysz (l. c.) has put forwards similar objections.

This classification arises from the circumstance, that the investigation of the first examined toxin, that of diphtheria, presents great difficulties. As it is usually kept in solution, it deteriorates comparatively quickly, and the curve of neutralisation of such a deteriorated toxin is very complicated. Besides the prototoxin which will be mentioned below, one phenomenon was especially conspicuous. A mixture of toxin with a comparatively great amount of antitoxin, injected on guinea-pigs, showed quite special effects: paralysis and rapidly disappearing oedema, which in guinea-pigs could not be produced by toxin alone. On the other side it was not possible, even when large amounts were injected, by means of this mixture to produce the typical effect of the toxin: acute death of the animal in a few days, or a necrosis on the place of injection, if the dosis had not been great enough to kill the animal.

From the experiments of that time is was therefore an obvious conclusion, that these two actions which differed so much were not the consequence of only a quantitative difference, but that the diphtheria toxin really contained two different components, the toxin and the toxon.

Still the examination of a new diphtheria toxin¹) has recently given a curve of neutralisation quite similar to that of tetanolysin and it is of special importance, that this toxin in contrast to all those previously examined, produced paralysis quite as well, when it is injected alone in small amounts, as when its "toxones" (mixtures with antitoxin) are injected. We are therefore justified in concluding, that the toxones are not bodies,

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1) Dreyer and Madsen: Studies on diphtheria toxin.

different from the toxins, but that they are the same toxin, diluted in different degrees, the combinations of toxin and antitoxin probably having no action at all. With this we are far from maintaining, that all toxins are homogeneous. On the contrary certain preparations of diphtheria toxin give a curve of this form[1]).

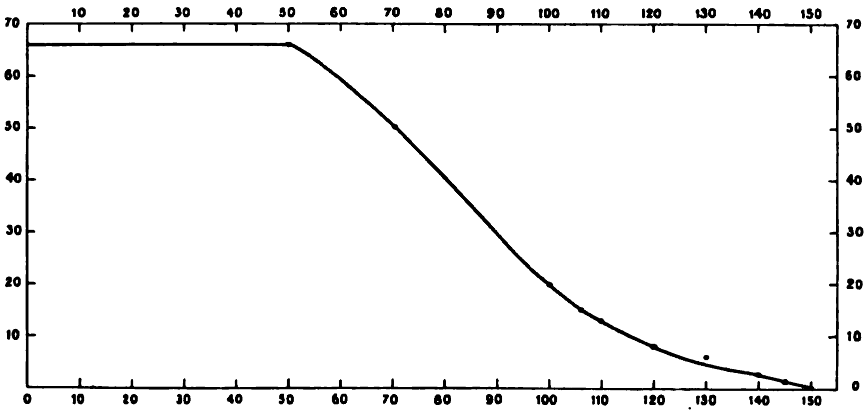

Fig. 7.

In this case it is the less toxic or perhaps the quite atoxic part of the toxin, which is first neutralised by antitoxin. The supposition seems necessary, thas this preparation of diphtheria toxin contains two elements, capable of fixing the antitoxin and of which the one with the greatest chemical affinity hardly possesses any toxic properties (Ehrlichs proto-toxoid), while the other (the toxin s.s.), allthough chemically weaker, is highly toxic.

A somewhat analogous case seems to be that of normal serum, described above; in small doses this diminishes the toxic effect in the same manner as other proteids, in greater doses it has a similar action as antitoxin. The most simple supposition is, that in the normal serum containing several different kinds of proteids, is also present a little amount of a body with neutralising action upon tetanolysin, while the usual proteids only to a certain degree decrease the action of tetanolysin.

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[1]) Th. Madsen: Om Difterigiftens Konstitution. Overs. v. D. kgl. Danske Vidensk. Selskabs Skrifter 1899.

In the case of chemical processes of dissociation, K is changed with the temperature and also in this case the decomposition of the combination toxin-antitoxin increases with increasing temperature as in most of the phenomena of dissociation. By means of a quantitative investigation of this change it is possible *to determine the amouns of heat (W) set free, when a grammolecule of toxin combines with a grammolecule of antitoxin.* For this the formula of van't Hoff is valid:

$$\frac{d \log. nat. K}{dt} = \frac{W}{1{,}99\, T^2}$$

For the purpose of this determination experiments with the same toxin and antitoxin were made at 20^0 and 37^0 C. All the concentrations used at the low temperature were double as great as at the high temperature and the experiments at the low temperature were undertaken once during 6 hours, another time during 7,5 hours, while the test tubes at the high temperature were kept a $37{,}2^0$ C. for only one hour and then were put into the ice safe.

As the experiments give good illustrations of the exactitude, which can be obtained, following details shall be communicated. The series of experiments at 20^0 C. were carried out with solutions of toxin which were 0,2 % and 2 %, and which per cc. contained the following amounts of a $^1/_{80}$ % antitoxin solution: 0,1, 0,2, 0,4, 0,7 and 1 cc. The relative toxicity of the different solutions, containing the same amount of toxin was determined through the comparison of the concentration of the solutions, which had effected the same degree of hæmolysis; the toxicity of the solution, free of antitoxin, was used as unit.

In the following table under the heading *a* the amounts of antitoxin added are indicated, and the other headings indicate the degree of colour of the solution, used for the comparison.

Tab. 10.

The relative toxicity of mixtures of toxin and antitoxin at 37° C.

Experim. 1.

a	12	9	5	4	3	Mean
0	1	1	1	1	1	1
0.1	0.74	0.84	0.75	0.61	0.63	0.71
0.2	0.48	0.48	0.49	0.43	0.43	0.46
0.4	0.27	0.256	0.30	0.28	0.32	0.29
0.7	0.19	0.22	0.23	0.21	0.23	0.22
1.0	0.13	0.13	0.19	0.17	0.18	0.16

Tab. 10 a.

Corresponding experiment at 37,5° C.

Experim. 2.

a	12	9	6	5	3	Mean
0	1	1	1	1	1	1
0.1	0.63	0.63	0.58	0.50	0.50	0.57
0.2	0.50	0.50	0.50	0.50	0.46	0.49
0.4	0.33	0.33	0.35	0.32	0.34	0.33
0.7	0.23	0.23	0.25	0.22	0.20	0.23
1.0	0.12	0.13	0.14	0.17	0.18	0.15

III

Tab. 11.

Corresponding experiment with double concentration at 20° C.

Experim. 3.

a	18	10	7	5	3.5	Mean
0	1	1	1	1	1	1
0.1	0.36	0.46	0.50	0.53	0.53	0.48
0.2	0.17	0.19	0.20	0.21	0.28	0:21
0.4	0.09	0.11	0.13	0.13	0.15	0.12
0.7	(0.042)	0.054	0.066	0.078	0.100	0.065
1.0	(0.030)	(0.036)	0.040	0.042	0.048	0.040

Table 11 a.

Same experiment at 20°.

Experim. 4.

a	16	12	8	6	4	Mean
0	1	1	1	1	1	1
0.1	0.57	0.43	0.49	0.50	0.37	0.47
0.2	0.42	0.31	0.42	0.36	0.25	0.35
0.4	0.18	0.14	0.16	0.19	0.16	0.17
0.7	0.054	0.073	0.072	0.088	0.075	0.068
1.0	(0.025)	(0.025)	0.036	0.036	0.032	0.031

The ciphers betwen brackets are extrapolated by comparison with the next ciphers in the table.

It appears from comparison of the different experiments, that the results of the experiments at 37° C show a very great mutual agreement, when we except the ciphers for 0,1 antitoxin. Thus the errors of experiment at the low temperature are much greater. By formation of new means the following values of x are obtained, besides which is given the value x calc., which as above indicated is calculated from the formula

$$x\,[np - (1-x)] = K\,(1-x)^2$$

where n is the amount of antitoxin (0,1—1,0) and x the toxicity; p was found = 6,0. K at 37,25° C = 1,18, at 20° C = 0,25

Table 12.

n.	x accord. to		mean		
	exp. 1	exp. 2	x obs.	x calc.	
0	1	1	1	1	
0,1	0,71	0,57	0,64	0,640	
0,2	0,46	0,49	0,475	0,477	t = 37,25
0,4	0,29	0,33	0,31	0,319	T = 310,25
0,7	0,22	0,23	0,225	0,214	
1,0	0,16	0,15	0,165	0,161	
0	1	1	1	1	
0,1	0,48	0,47	0,475	0,514	
0,2	0,21	0,35	0,280	0,276	t = 20,0
0,4	0,12	0,17	0,145	0,128	T = 29,3
0,7	0,050	0,068	0,067	0,067	
1,0	0,040	0,031	0,036	0,045	

The concordance is in both cases very good, especially at the high temperature. According to log. nat. 1,18 = 2,3025 . 0,0719 and log. nat. 0,25 = 2,3025 (0,3979—1) from the above formula the following value of W is obtained

$$W = \frac{2{,}3025\,(1{,}0719 - 0{,}3979)}{310{,}25 - 293} \,.\, 293 \,.\, 310.25 \,.\, 1{,}99 = 6600 \text{ cal.}$$

Through the combination of one grammolecule of tetanolysin with one grammolecule of antitetanolysin is thus set free an amount of heat = 6600 cal., which cipher probably is right within 600 cal. The heat-reaction is thus rather strong from a chemical point of view, the heat developed being about the half of that set free by the neutralisation of a strong acid with a strong base (13700 cal. at 18° C.).

It is noticeable, that the constant in the above equation, which from experiments from may 1900 was found 0,115 at 37° C, now the first of july 1902 had a value, ten times as great, 1,18 at 37,25° (corresponding to 1,15 at 37°). The power of the toxin to combine with antitoxin has thus considerably decreased during 2 years. Most probably it is the toxin, which has changed. According to the experiments from may 1900 2 cc. of a 2 % tetanolysin solution were then equivalent to 0,276 cc. of a $^1/_{40}$ % solution of antitoxin. According to the experiments from year 1902 2 cc. of a 2 % tetanolysin solution were equivalent to one sixth of two cc. $^1/_{80}$ % antitoxin solution, that is — 2 cc. 2 % tetanolysin solution were equivalent to 0,167 cc. $^1/_{40}$ % antitoxin. The power possessed by this preparation of tetanolysin of fixing antitoxin has during these two years decreased to six tenth of its value in 1900. It seems very improbable, that the power of the antitoxin has increased; it is much more likely, that it has kept constant. It must therefore be supposed, that the tetanolysin has become considerably weaker during these two years owing to that about 40 % of the molecules of toxin have been decomposed. The remaining molecules of toxin have also changed, so that the constant K increased to ten times as much; still this change is not very essential, as it corresponds to the change of the toxin, arising from an increase of temperature of 26° C. Besides the hæmolytic power of the toxin has decreased during the same time, so that in 1902 a dose about three times as great as that required in 1900, was necessary to produce a certain degree of hæmolysis.

As it was of interest to compare this decrease with that, which occurs much quicker in solution, especially if this is diluted, experiments were undertaken to compare the strength of fresh toxin with that of an older one, which had been kept in solution during 4—5 days. The latter solution was 2 % and made by mixing several older ones together. From standing at 20° C. (temperature of the room) the toxicity of the solution had decreased to about $^1/_6$ of the original strength, it had thus decreased about double as much as the solid tetanolysin during two years.

The experiments were rendered very difficult for this reason, that the blood corpuscles in this solution of lysin turned violet, indicating commencing putrefaction. The experiments with fresh toxin gave the same results as those just mentioned (at 37°), allowing for errors of experiment. The blood was diluted with 0,85 % NaCl solution.

The experiments with old lysin gave the following results (the table is arranged as the previous ones). The indices of x signify the degree of colour, used for comparison.

Although the colour was not very suitable for the comparison, the result agreed very well with the calculation, which gave $p = 6$ and $K = 1,74$.

The fact that p has the same value as in the case of fresh tetanolysin shows, that in this case (in contrast with the preceding, where tetanolysin was kept dry) no antitoxin fixing molecules of toxin have been decomposed. Still the hæmolysing power of the toxin has

Table 13. The action of old tetanolysine.

n	X_{15}	X_{10}	X_6	X obs.	X calc.
0	1	1	1	1	1
0.05	0.81	0.76	0.80	0.79	0.79
0.1	0.57	0.67	0.68	0.64	0.67
0.2	0.50	0.57	0.68	0.58	0.53
0.3	0.36	0.35	0.47	0.40	0.44
0.5	”	0.35	0.36	0.35	0.33
1.0	”	0.19	0.22	0.20	0.21

decreased to about one sixth, which shows that a transformation of the molecules of toxin has taken place. This probably depends on a very little change of the molecules of toxin, perhaps a transformation to a metameric compound, less toxic. To examine this an investigation of the time, taken by this process in the case of toxins of different concentrations would be of great interest.

By this transformation the constant of dissociation, K has only been influenced to a small extent, having increased from 1,18 to 1,74, viz. by about 50 %. This circumstance brings about, that by the same amount of antitoxin the toxicity of the old tetanolysin is not diminished in the same proportion by the same amount of antitoxin as the fresh tetanolysin. According to the calculated ciphers, without doubt much less faulty than those directly observed, the toxicity of fresh and old lysin with the following additions of antitoxin — is diminished to the values indicated in the second and third columns (Tp. 37° C.).

Tab. 14.

Comparison of old and fresh tetanolysin.

n	x (fresh lysin)	x (old lysin)	diff. in percent
0	1	1	0,0
0,1	0,640	0,674	5,3
0,2	0,477	0,528	10,7
0,4	0,319	0,378	18,5
0,7	0,214	0,269	25,7
1,0	0,161	0,209	28,5

If we, according to the usual view (Ehrlich), try to explain the decrease of the toxicity of tetanolysin through the supposition, that the toxin is to a certain extent (in this case to $^5/_6$) transformed to an atoxic body (proto-toxoid), it seems to be an obvious explanation of this fact, that the proto-toxoid has a greater affinity to the antitoxin than the toxin itself. In this supposition a greater percentage of the proto-toxoid is fixed to begin with than later on. In the present case the difference of K for fresh and old lysin is certainly not especially great, but in other cases this difference might be very pronounced, so that it for this reason might seem necessary to suppose the formation of a proto-toxoid. An analysis such as that above for tetanolysin will show, if the supposition is necessary or not.

As above indicated the toxin does not combine immediately with antitoxin, but this phenomenon is especially obvious, when the amount of antitoxin is great, as appears from the following experiment.[1] The result is given in the table below, indicating how many cc. of the mixture, — containing per cc. 1 milligram of lysin and n parts $^{1}/_{40}$ % antitoxin — are necessary to produce a certain degree of hæmolysis (7). It appears from the table that the amount necessary (the "toxic quantity") increases with the time, which elapsed after the mixture of toxin and antitoxin, kept at 20^{0} C.

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[1] Th. Madsen: Sur les toxones. XIII. Congres international de médecine. Paris 1900.

n	minutes						
	10	20	30	60	120	240	1440
0	0.23	„	0.23	„	0.23	„	„
0.1	0.33	0.33	0.33	„	0.33	„	„
0.2	0.50	0.57	0.67	0.67	0.67	„	„
0.5	1.6	2.0	2.0	2.3	2.3	„	9.0
1.0	1.7	2.4	3.8	3.5	4.5	5.0	„
1.3	2.0	2.6	3.2	7.3	5.6	6.4	„
1.6	2.3	3.3	3.7	5.4	5.8	7.0	„
2.0	2.5	3.0	4.2	5.4	7.0	9.0	„

It appears from these ciphers, that the toxicity of the toxin-antitoxin mixture continually decreases with the time. In the case of mixtures, containing small amounts of antitoxin (n=0.1) this decrease — that is the from the table apparent increase of the "toxic quantity" viz. the dose, necessary to produce the given degree of hæmolysis — seems to have finished even before the first time of observation viz. 10 minutes after the mixture. At the time 0 all the experiments ought to give the same value, viz. 0,23.

In the case of greater additions of antitoxin (n=0,5) the dose necessary increases rapidly with the time within the observed interval of time — with about 50 % between the time t = 10 and t = 60. (It probably depends on errors of experiment, that the ciphers in the case $n = 0{,}2$ increase so much with the time after 10 min.) The observation after 24 hours ($t = 1440$) shows that after the decrease just mentioned a continual decrease takes place, caused by the transformation of the lysin. It is therefore necessary not to use too long times of observation if the process here examined is not to be interfered with through a deterioration of the toxin itself. The experiments with $n = 0.1$ and 0.2 show that this does not take place, if the time be limited to 120 minutes. The greater the addition of antitoxin, the greater is the relative increase for the toxic quantity necessary within the specified space of time.

A new series of experiments was undertaken at constant temperatures (6° C and 24.5° C) and the time for the experiments was limited to 125 minutes at the low temperature and 50 minutes at the high. The amount of antitoxin added was taken from the interval $1._0 - 2._0$, which had shown the smallest variations.

According to the above equation of equilibrium between toxin, antitoxin and toxin combined it seemed probable, that the velocity of reaction could be expressed by the following formula.

$$\frac{dx}{dt} = K_1 (A - x)(B - x) - K_2 x^2$$

where A and B stand for the amounts of toxin and antitoxin added in the beginning, and x signifies the amounts of these bodies, which are combined together. $K_1 : K_2$ should then be of equal value to the dissociation constant K, calculated from the above experiments. But it appeared to be quite impossible to include the experiments in this formula, and the problem was therefore treated quite empirically. A practical limit (I) for the reaction was found by allowing it to continue for a long time (400 to 450 minutes); a moderate equalization is applied to these values.

For the calculation of the amounts A and B this limit was taken as point 0 and the velocity of reaction determined according to the formula.

$$\frac{dx}{dt} = K\,(A - x)^{a}\,(B - x)^{b}$$

a and b being exponents, the values of which were determined from the experiments. The toxicity at the time 0, viz. that of pure tetanolysin was put like 34.3, 0.3 cc. of this solution being necessary to produce the given hæmolysis (7), when the experiments had been kept at 37° C during 1 hour (2,5 $^0/_0$ blood). The toxicity of all the solutions used for the experiments has been determined in this way. In the next tables the toxicity (A — x) has been calculated from the supposed equilibrium (I) taken as zero, viz: if this (I) for instance corresponds to the toxicity 1, then A — x = 7 signifies, that the toxicity, really observed was 8, that is that 1. 43 cc. of the solution in question was necessary to produce the given hæmolysis. Still this mode of calculation is not applied to the ciphers observed in the case of the longest time of reaction, which were exclusively used for the determination of I.

The calculated figures are found under the supposition $a = 2$, and $b = 0$, viz. that the differentialequation for the velocity of reaction had this form:

$$\frac{dx}{dt} = K\,(A - x)^{2}$$

or integrated:

$$\frac{1}{A - x_2} - \frac{1}{A - x_1} = K\,(t_2 - t_1)$$

III

Change of the toxicity with the time of 6° C.

Tab. 16.

n	0	10	20	30	55	125	400	I
1.0	32.8	7.5	4.5	„	2.8	1.1	1.5	1.5
1.3	33.2	6.1	4.9	„	2.3	1.3	1.2	1.1
1.6	33.5	7.0	5.2	3.1	2.2	1.5	1.0	0.8
2.0	33.7	7.1	3.9	3.1	2.0	1.3	0.6	0.6
Mean	33.3	6.9	4.6	3.1	2.4	1.3		
Calc.	33.3	7.4	4.2	2.9	1.6	0.8		

In the present case K was put = 0, 0 104 expressed by the above indicated arbitrary unit for the amount of toxin, the unit of time was 1 minute.

When the observations at 24.5° are treated in the same manner, the following results are obtained.

$A-x_0$ varying between 32,8 and 33,9 being relatively great, $\frac{1}{A-x_0}$ becomes so small a figure, that it without recognizable error can be considered as constant in all four series. It follows from this, that all values of x_1 must also be almost constant; the same applies to all

The change of the toxicity with the time at 24.5° C.

Tab. 17.

n	0	10	20	30	50	180	450	I
1.0	33.3	2.0	1.3	0.8	0.5	0.1	1.1	1.0
1.3	33.5	2.1	1.2	0.7	0.5	0.1	0.8	0.8
1.6	33.7	2.0	1.2	0.7	0.5	0.2	0.8	0.6
2.0	33.9	1.8	1.0	0.8	0.6	0.3	0.6	0.4
Mean	33.6	2.0	1.2	0.75	0.5	0.2		
Calc.	(33.6)	2.1	1.1	0.72	0.48	0.13		

values of x_2 and so on. The above tables show this also to be the case, allowing for errors of experiment. And it is caused by this circumstance, that the differentialequation for the velocity of reaction has obtained the form, indicated above.

In the later series K stands for = 0,045. It follows from this that with an increase of the temperature of 18,5° C K increases in the proportion 1:4,3. This corresponds to an increase of K in the proportion 1:2,2 with an increase of temperature of 10° C.

The variation with the temperature of this velocity of reaction is thus of the same magnitude as that of the other velocities of reaction, dealt with above, as well as of the velocity of chemical processes generally.

Although the above equation is exceedingly simple and of a quite empiric nature it shows at 24,5° (the temperature of the room during the experiment) an excellent agreement between calculation and observation. At 6° they do not agree so well; the velocity of reaction seems to decrease to some extent with the increase of time. This is probably owing to the temperature of the ice safe not having been completely constant — it varied rather irregularly between 4,5° and 7,0° C during the time of experiment Besides it is practically impossible to avoid heating the solutions while handling them, this caused the velocity of reaction to be greater at the beginning than later on.

It may also be that the empiric relation is not valid with the same exactitude for this long times of reaction (t = 125 min).

At all event this relation is very curious from a chemical point of view and it is rather useful for practical purposes. The most curious thing about it is, that it is true of the reaction from the very beginning, although at the beginning of the experiments themselves three quarts of the reacting amounts are transformed.

From a theoretical point of view the above relation indicates, that during the action of toxin upon antitoxin two molecules of the former attack an exceedingly small fraction of the latter (according to the formula o). The last mentioned circumstance could be explained thus, that the molecules of antitoxin are to a very great extent composed of great aggregates of molecules, which do not to an appreciable degree react with the toxin. The molecules of toxin should then combine with only a very small part, y, of the molecules of antitoxin, this being in chemical equilibrium to the other according to the formula

$$y^n = (B - x - y)$$

or

$$y = (B - x - y)^{\frac{1}{n}}$$

in which n is a very great figure.

On the other hand from the equilibrium, mentioned above, the conclusion was drawn, that during the reaction between toxin and antitoxin one molecule of the former combines with one molecule of the latter forming two molecules of combination. This conclusion, which is based upon the mechanical theory of heat, is undoubtly much more probable than the latter, based upon kinetical views. It must be remembered, that this is not the first time, velocities of reaction behave differently from what we should expect from the chemical formula of reaction.

The equation given above is still of so peculiar a form that a thorough investigation of its signification would probably be of great interest. Something analogous to the combination between an acid and a base cannot of course be expected for the reason that the combination between these bodies takes place so quickly that it is impossible to follow the course of time taken by the reaction.

IV.

SVANTE ARRHENIUS AND THORVALD MADSEN

ON THE MOLECULAR WEIGHT OF DIPHTHERIA TOXIN.

A PRELIMINARY NOTE.

The preparations of toxin and antitoxin hitherto produced have been far from pure, so that it is impossible to indicate their composition and especially their percentage of active bodies. Under such circumstances we cannot at present hope to obtain any idea of the molecular size of the active bodies by means of direct methods, as determination of the freezing or boiling point or of the vapour-pressure of solutions of toxins and antitoxins.

We have therefore tried how far we could get by means of an indirect method. By studying the diffusion of the bodies in question we are able to obtain some knowleadge of their molecular weight. The velocity of diffusion of gases through a porous wall being inversely proportionate to the square root of their molecular weight, Pickering tried if the same would not be the case for fluids diffusing in water. From his values that differ very much from those of other authors he concluded, that the dissolved molecules do not behave as gas-molecules. Notwithstanding, this rule that the velocity of hydrodiffusion is in inverse proportion to the square root of the molecular weight, has later on been tested by Hüfner and Euler, and has been found right as regards the main points; still it does not apply to ions. It k denotes the velocity of diffusion, m the molecular weight, then at

12^0 C the product $k \sqrt{m}$ is 5.4 (mean value for cane sugar 5.3, mannite 5.4, citric acid 5.7, tartari cacid 5.4, ethyl alcohol 5.0 and ammonia 5.8).

We have therefore compared the velocity of diffusion of diphtheria toxin and some other bodies with a substance of a well determined molecular weight and from these facts we tried to get an approximate value of the constant of diffusion of diphtheria toxin.

As we supposed this to be very small we considered a long time of diffusion to be necessary; as besides the specific weight of the solutions does not perceptibly vary with the percentage of toxin which they contain, it was impossible to employ the usual method: to let them diffuse in their ordinary vehicle of solution, the water. We therefore have permitted them to diffuse in solutions of gelatine.

A 5 $^0/_0$ solution of french gelatine was prepared, neutralised with soda and sterilised; of this solution 15 cc. were poured into sterilised test tubes, whereby a gelatine pillar about 10 cc. high was obtained. The test tubes were put into an ice safe in a vertical position and allowed to get stiff. Then 2 cc. of an aqueous solution of the body to be examined was poured by means of a pipette over the gelatine pillar and the tube was closed with a sterilised cork. It was thereafter allowed to stand in the ice safe for a certain time, during which the diffusion was going on. After this time the test tube was taken up, the fluid covering the gelatine removed, and the tube over the gelatine quickly washed with distilled water. The tube was cautiously crushed and the surrounding glass thus removed from the gelatine pillar. This was then cut to pieces of a certain length, whose weight was found. The toxicity of the fluid above and of the pieces was found by means of animal experimentation. As a comparison experiments were made with solutions of sodium chloride, which were titrated.

In the case of 1 n sodium chloride at 6° C we found in this way the coefficient of diffusion 0,95, while this according to Graham ought to be 0,801 and according to Oeholm 0.75. Our value is thus 26.6 % to great. This arises from the mode of experimentation, the fluid covering the gelatine pillar being throughout of a similar concentration, while this in the case of the usual mode of experimentation should be smallest where it touches the gelatine pillar. Also the presence of gelatine effects some deviation from the usual course of diffusion. As easily will be seen the correction is ever the same, if the diffusion has proceded to the same degree. In different cases the correction must only differ but very little, we therefore calculate the velocity of diffusion in pure water from the value found after the related method by dividing this value with 1.266.

On the other side the velocity of diffusion increases with the temperature with about $^1/_{30}$ of its value at 12° C. for every degree. The velocity of diffusion is therefore 1.25 times as great at 12° C. as at 6° C., the temperature of the ice safe. The velocity of diffusion in water at 12° C. is therefore $1.25 : 1.266 = 0.986$ times greater as the determined value.

In the case of experiments with diphtheria toxin the height of the fluid covering the gelatine was 1.3 cm. and the pieces into which the gelatine pillar was divided were of a similar height.

The dosis minima lethalis for guinea-pigs of 250 grams weight was determined for the original toxin and for the contents of the test tube; the following results were obtained:

	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]

[illegible]

[illegible]

[illegible]

[illegible] = [illegible]
[illegible] = [illegible]
[illegible] = [illegible]
[illegible] = [illegible]

[illegible]

in question, these constants could hardly with our present knowledge be used for an exact determination of their molecular weight. In the mentioned equation K $\sqrt{m}$ = 5,4, 5,4 is really no constant, but increases for decreasing molecular weight (or rather, when the molecules become less complicated). According to Euler in the case of Cl_2 this value is 10,2. But when a thorough investigation of the signification of the constant of difsion for bodies with a high molecular weight has been made the mentioned method probably will be usefull in the case of toxins and antitoxins.

V.

GEORGES DREYER AND THORVALD MADSEN

STUDIES ON DIPHTHERIA TOXIN.

In some former papers dealing with the study of diphtheria toxins derived from various strains of bacilli, we have considered the relation of toxin to antitoxin.

During the continuation of these researches we have met with a toxin, that is both very remarkable and in many ways very different to the toxins examined before by Ehrlich and Madsen.

Although the experiments in connection with the thorough analysis of this toxin are not yet completed, we think it of interest to communicate some of the results at the present time, since they are quite in accordance with those obtained in the investigations on Tetanolysin mentioned in the preceding paper.

The toxin was obtained in the following way: 20 litres of bouillon, prepared according to the directions of Dean, were inoculated with a strain of diphtheria culture Park & Williams (No. 8) given to us by Dean. After inoculation the bouillon was left in the incubator at 36° for a period of fourteen days ($^{15}/_{10}$—$^{28}/_{10}$ 1901). The culture was then removed from the incubator, filtered through ordinary filter paper, bottled with a covering of Toluol and shaken daily for a fortnight. At the end of this time the toxin was quite clear and sterile and has so remained.

The toxin proved to be very strong. From numer-

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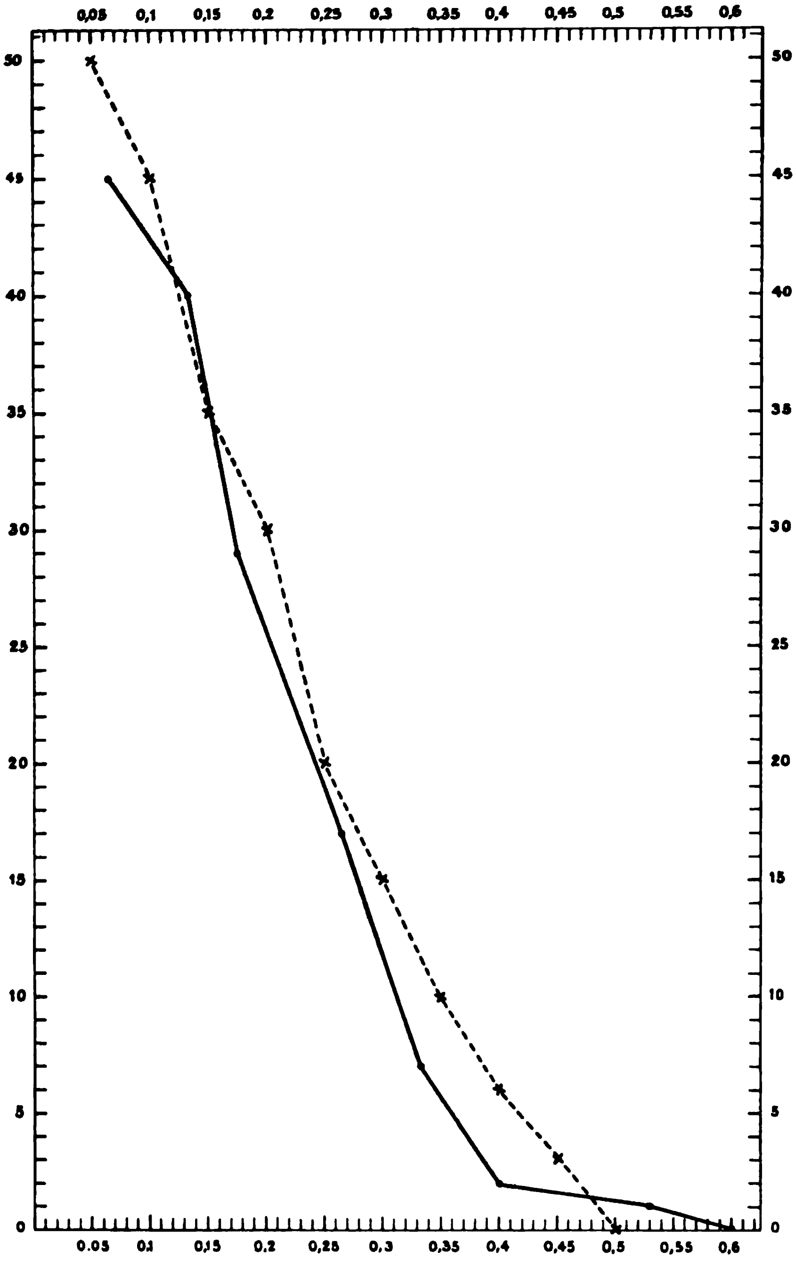
0,05
0,1
0,15
0,2
0,25
0,3
0,35
0,4
0,45
0,5
0,55
0,6
50
45
40
35
30
25
20
15
10
5
0

ous experiments on guinea-pigs of 250 Grs. weight, the minimal lethal dose was found to be 0.0015 i. e. guinea-pigs of the above weight were killed with certainty within 4—6 days by the injection of this amount of toxin.

Besides this the value of $L\dagger$ and L_0 were found by means of Ehrlich's test serum to be: $L\dagger = 0.2$ and L_0 ca. 0.05.

The determination of L_0 for this toxin was much more difficult than for any other previously examined, therefore the value 0.05 must be regarded as rather arbitrary.

In considering the above values for minimal lethal dose, $L\dagger$ and L_0, it will be noticed that the $L\dagger$ is four times the L_0 and that the $L\dagger$ and L_0 respectively contain 133 and 33 minimal lethal doses. These constants are entirely different to any that have been determined before for any other toxin.

For making the "spectrum" in the manner directed by Ehrlich, by partial saturation of the given quantity of toxin with antitoxin, 0.1 Cc. was chosen as the most convenient quantity of toxin for the purpose. As antitoxins the following were used: 1) a real antitoxin i. e. Ehrlich's test serum and 2) the normal serum from a non-immunised horse.

Much trouble has been taken to find out the history of this horse, so that it should be as far as possible quite certain that it had never been in contact with Diphtheria or been used for the production of diphtheria antitoxin.

The results obtained are represented by two curves, the dotted line indicates the curve obtained by the use of the test serum and the unbroken line that of the serum from the normal horse.

It will be seen that there is a very striking resem-

V

blance between these two curves, but on the other hand that they are very different to those found for the other toxins before examined, especially in the absence of a marked prototoxin zone.

It must be taken into consideration that the minimal lethal dose (T) was at the time of the first examination 0.0015 Cc. and at the present moment is 0.0027 Cc.

In looking at the curves it will be observed that there is a tendency to flatness in the upper portion. This seems to indicate that a formation of prototoxoids was probably taking place at the time of the examination of the toxin. We have therefore postponed giving a detailed account of the experiments until the curves had been examined again and at a time when it could be supposed that the formation of prototoxoid had advanced to a considerable degree.

One fact of great interest in connection with this toxin is, that contrary to all others previously examined it produced paralysis in guinea-pigs both after the injection of a non lethal dose of pure toxin and after a toxin-antitoxin mixture.

The above mentioned curves are closely analogous to those described in the preceding paper on Tetanolysin. We have thus been fortunate enough to find and to examine a diphtheria toxin in a state well suited for a thorough analysis.

The details of this will be published as soon as our experiments have been completed.

VI.

AXEL JØRGENSEN AND THORVALD MADSEN

THE FATE OF TYPHOID AND CHOLERA AGGLUTININS DURING ACTIVE AND PASSIVE IMMUNISATION

Among the means we have at our disposal for the study of the nature of the immunity, comparatively little attention is paid to the investigation of the increase and the decrease of antibodies during different modes of immunisation. From different points of view this is rather curious. From a theoretical one, because the study of this phenomenon seems to give valuable information about what is going on in the organism during the immunisation; from a practical point of view, because under the immense production of antitoxic sera, which is now going on the whole world over, it is of great economical interest to know as exactly as possible, at what moment the highest amount of antibody may be expected. It is a well known fact, that Brieger and Ehrlich[1]) showed the way by their demonstration of the undulatory course of the antitoxin in the case of a goat, immunised against tetanus. In a more extensive series of experiments Salomonsen and Madsen[2]) thereafter showed that phenomena quite analogous occurred in the case of horses

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[1]) Brieger und Ehrlich: Beiträge zur Kenntniss der Milch immunisirter Thiere. Zeitschr. f. Hygiene etc. XIII. 1893.

[2]) Salomonsen et Madsen: Recherches sur la marche de l'immunisation active contre la diphtérie I. Annales de l'Institut Pasteur 1897.

II. — — — 1899.

— — Om Forskelligheder i Serums antidiphtheriske Styrke hos aktivt immuniserede Heste. Nordisk Med. Arkiv 1897. Festband för Axel Key.

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immunised against diphtheria toxin, and Dean[1]) got similar results. The third research on these lines was made by Morgenroth[2]), who showed that the rennin immunisation followed an undulatory course like that of diphtheria and tetanus. Later on Bulloch[3]) demonstrated, that in the case of the hæmolysins similar phenomena occurred. The last antibody drawn under this kind of experiments is that of botulismus, studied by Forssman and Lundstrøm[4]), who found the antibotulismus curve very like that of diphtheria and tetanus antitoxin.

The difficulties in procuring an extensive and reliable material are mainly caused by the necessity of determining the antibodies by means of animal experimentation. For our investigations we therefore searched for such antibodies, the testing of which could readily be performed in test tubes and found the agglutinins very suitable for this purpose.

The following experiments are made with *typhoid* and *cholera agglutinin* and can be classified into three groups. In the first is treated the production of agglutinin during various kinds of active immunisation; in the second the fate of agglutinins, introduced in the organism (passive immunisation). At last we have examined how an animal passively immunised reacts during attempts on active immunisation.

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[1]) **George Dean: Problems of diphtheria immunity. Transact of the Pathol. Society of London. 1900.**

[2]) **J. Morgenroth: Ueber den Antikörper des Labenzyms. Centralblatt f. Bact. etc. XXVI. 1899.**

[3]) **William Bulloch: On the nature of hæmolysis and its relation to bacteriolysis. Transact. of the Pathol. Society of London. 1901.**

[4]) **Forssman et Lundstrøm: Sur la marche de la courbe d'antitoxine dans l'immunisation active contre le botulisme. Annales de l'Institut Pasteur 1902.**

VI

THE MODE OF PROCEEDING.

As none of the earlier methods was exact enough for our purpose, we performed the testing of the agglutinating power by the macroscopic precipitation in test tubes in the following manner.

The culture of Bacillus typhosus, which served for all our experiments, had been cultivated on artificial media in the laboratory for several years and was only slightly virulent. 5 cc. of a 24^h broth culture injected on rabbits, and 100 cc. injected on goats, produced only a very slight illness, but was not at all able to kill the animals. A very light and clear broth was used for the culture, which was left 18—24 hours in the incubator. It was then carefully examined as regards infection with other microbes, and afterwards formaldehyd to $^1/_5\,^0/_{00}$ was added. This made it possible to keep the culture in uncovered test tubes for some hours at 37^0 without being contaminated.

A large series of test tubes was filled with exactly the same quantity of this fluid. For our first experiments 10 cc. were used, but it was often found more practical to take only 1,5 cc. in thin test tubes (11 × 70 mm.), especially when only small quantities of serum were at our disposal.

When we had to test and compare the agglutinating power of for instance 12 different sera, then for each test a series of such tubes was used and decreasing amounts of the serum were distributed among them. The tubes from all 12 tests having been thoroughly shaked were at the same time placed alltogether in an Ostwald water incubator at 37^0 for about 1$^1/_2$ hour, then at once taken out and examined.

If suitable doses of serum were chosen, each series will show a continuous scale of clarification, from complete precipitation in the test tubes with the greatest amount of serum, through decreasing agglutination with finer and finer clumps until no agglutination at all can be observed. A test tube, with a not too advanced agglutination, will easily be distinguished from its neighbours through the size of its clumps. Such a test tube was taken out and as quickly as possible used for the determination of the tube in each of the other series which showed the corresponding degree of agglutination.

That, which was obtained in this way, was the *relation* between the agglutinating power of the sera to be tested, and just this relation was of importance for the following experiments. A control was usually made by repeating the comparison with another test tube containing a litte more agglutinin.

This way of determination is based upon a similar principle as the colorimetric method, used for measuring hæmolytic toxins[1]). By some practise rather exact results are easily obtained.

The most convenient way to determine the amount of agglutinin would be to keep the test tubes at exactly 37° for just the same time and always use the same degree of clarification as a standard. But as the conditions for the agglutination depend upon many circumstances which cannot be regulated, it was found more practical to have a standard agglutinin and always use the same dose of it for the test.

The following example will give the best idea of the mode of proceeding.

A rabbit, treated with increasing doses of B. typhos. culture, got on the $^{29}/_3$ an injection of such a culture and the following days until the $^{23}/_4$ the variations of agglutinin in its serum had to be examined. 12 samples of blood were taken.

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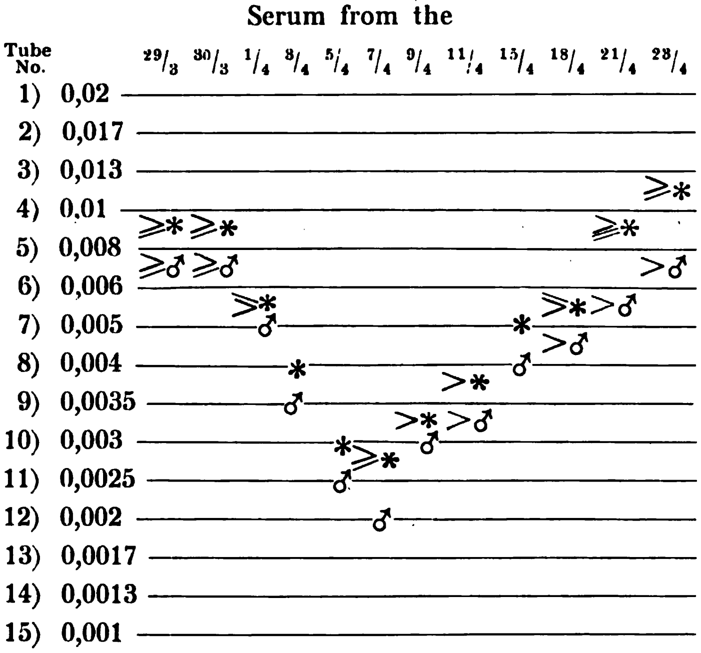
[1]) Th. Madsen: Ueber Tetanolysin. Zeitschr. f. Hygiene etc. XXXII. 1899.

Standard Serum.

Tube No.	
1)	0,1
2)	0,08
3)	0,06
4)	0,05 = *
5)	0,04 = ♂
6)	0,035
7)	0,03
8)	0,025
9)	0,02
10)	0,017
11)	0 (control)



For testing the standard serum 10 tubes were used, to which was added from 0,1 cc. to 0,017 cc. serum. For the testing of each of the other sera 15 tubes were used containing from 0,02 to 0,001 cc. Having been kept in the incubator for 1$^1/_2$ hour at the same time all the series were taken out, and tube no. 5 of the standard serum series (containing 0,04 cc.) was used for the testing (in the table indicated ♂). Its degree of clarification was found lying between that of no. 5 and no. 6 of the serum $^{29}/_3$, near up to no. 6, and it was judged to correspond almost to the action of 0,0065 cc. The serum $^{30}/_3$ was exactly like $^{29}/_3$, but the $^1/_4$ was stronger, as 0,005 cc. of this corresponded to 0,04 cc. of the standard serum etc. Afterwards tube no. 4 of the standard serum (0,05 cc.) was used for the testing (*), and the corresponding degree of agglutination determined for all the samples.

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For the purpose of making the following experiments more conspicuous, the action of the agglutinin is not indicated by the doses themselves, but by their reciprocal values, as it is usual in the standardising of diphtheria antitoxin. For the agglutinin we have thus introduced a unit, which is quite arbitrary and only of value for the comparison of our experiments.

The above example therefore is to set up in the following scheme.

Serum from the	0,04 cc. of the standard serum ♂	recipr. value	0,05 cc. of the standard serum *	recipr. value
$^{29}/_{3}$	0,0065 cc.	154	0,0085 cc.	118
$^{30}/_{3}$	0,0065 „	154	0,0085 „	118
$^{1}/_{4}$	0,005 „	200	0,0057 „	176
$^{3}/_{4}$	0,0035 „	286	0,004 „	250
$^{5}/_{4}$	0,0025 „	400	0,003 „	333
$^{7}/_{4}$	0,002 „	500	0,0026 „	385
$^{9}/_{4}$	0,003 „	333	0,0032 „	313
$^{11}/_{4}$	0,0033 „	303	0,0037 „	270
$^{15}/_{4}$	0,004 „	250	0,005 „	200
$^{18}/_{4}$	0,0046 „	217	0,0057 „	175
$^{21}/_{4}$	0,0056 „	179	0,0081 „	124
$^{23}/_{4}$	0,007 „	143	0,011 „	91

The sum total of the results of such series of tests is rendered most plain when plotted out on paper in the form of a curve. As abscissa is marked out the day, upon which the serum was taken, and as ordinate is indicated the agglutinin units pr. cc. of the serum.

The result of the experiment mentioned above is represented on curve No. 1. This is one of the first undertaken; with some practise more exact figures can be obtained.

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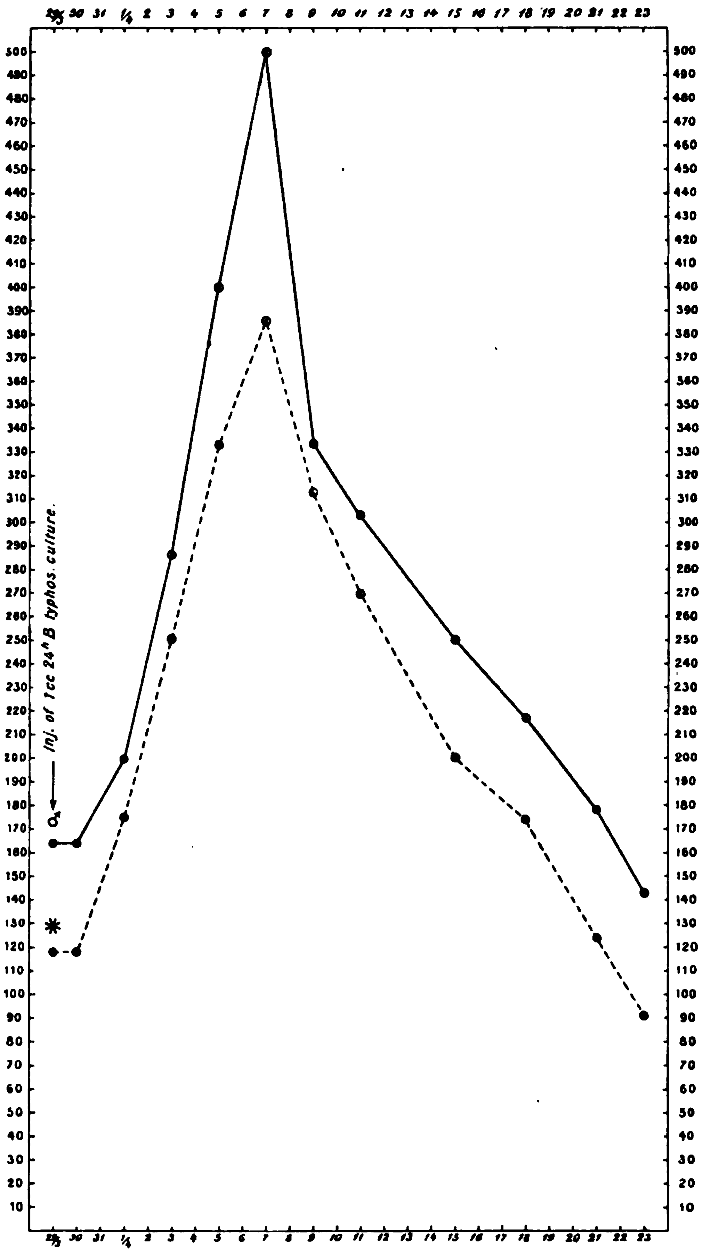
29/3
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31
1/4
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500
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290
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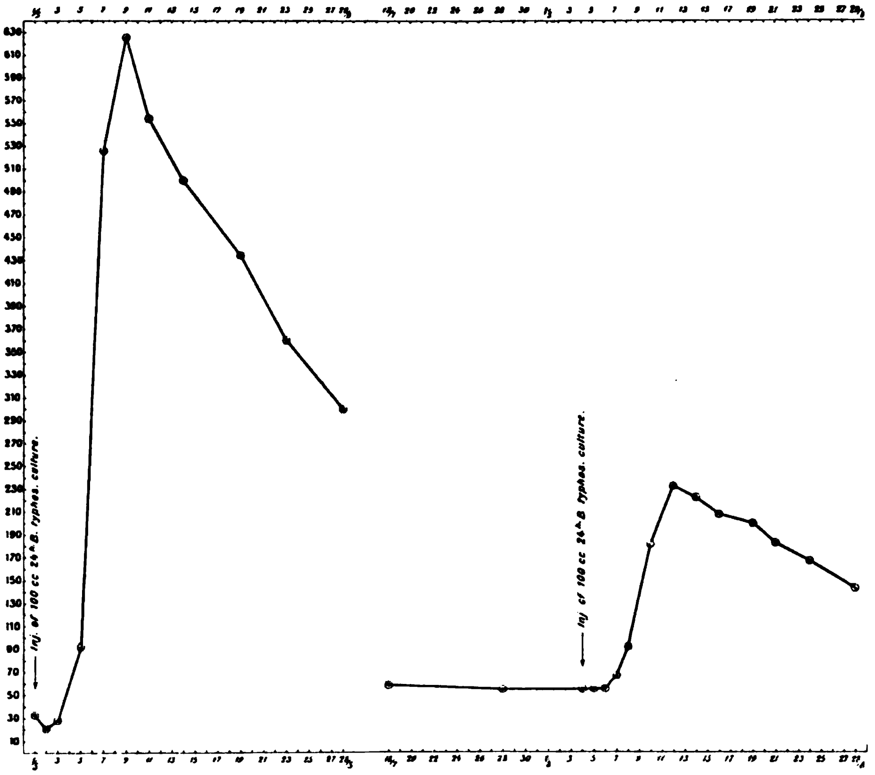
270
260
250
240
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
Inj. of 1cc 24^h B typhos. culture.
♂
✱

Curve No. 1

The experiments with Vibrio choleræ were undertaken in quite analogous manner, the only exception being that to each tube of culture 0,3 cc. ascites fluid was added.

ACTIVE IMMUNISATION.

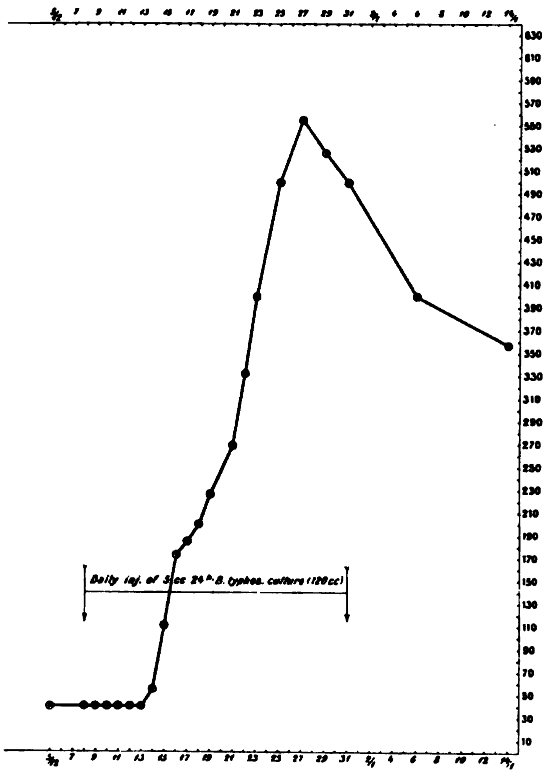
The object for our first experiments was to examine the oscillations in agglutinating power which occurred in the serum of actively immunised animals after a single large injection of a fresh culture of Bacillus typhosus resp. Vibrio choleræ.



Curve No. 2a

Rabbits and goats were used, which were previously immunised with 24h—48h cultures in small and increasing doses; the former intraperitoneally, the latter subcutaneously. During the experiments small amounts of blood were withdrawn from the ear vein of the rabbits and the jugular vein of the goats.

Of such experiments a great number was made which gave very uniform results. Examples are indicated on the curves 1—4.



Curve No. 2b

Curve No. 2a represents a goat which during several years had been immunised with B. typhos cultures. On the $^1/_5$ 1900, when the agglutinating power of the serum was 33, 100 cc 24h B. typhos. culture were injected. The next day showed a decrease to 22, but on the following days an increase first slow and then rapid was observed, until the maximum was reached the 9th day after the injection; thereafter a continual fall occurred.

For the next experiment (curve 3) served a goat, immunised with Vb. choleræ cultures, whose agglutinating

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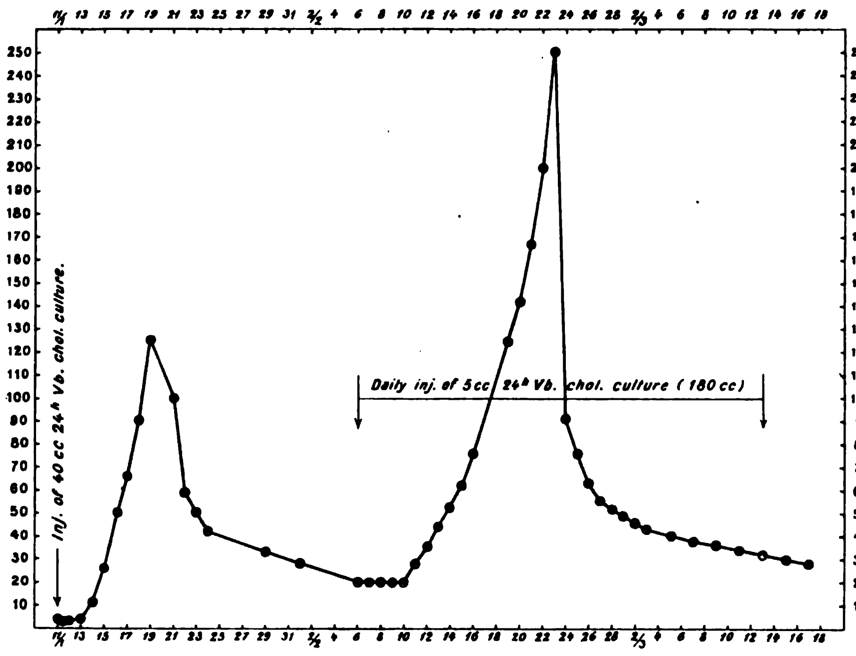


Curve No. 3

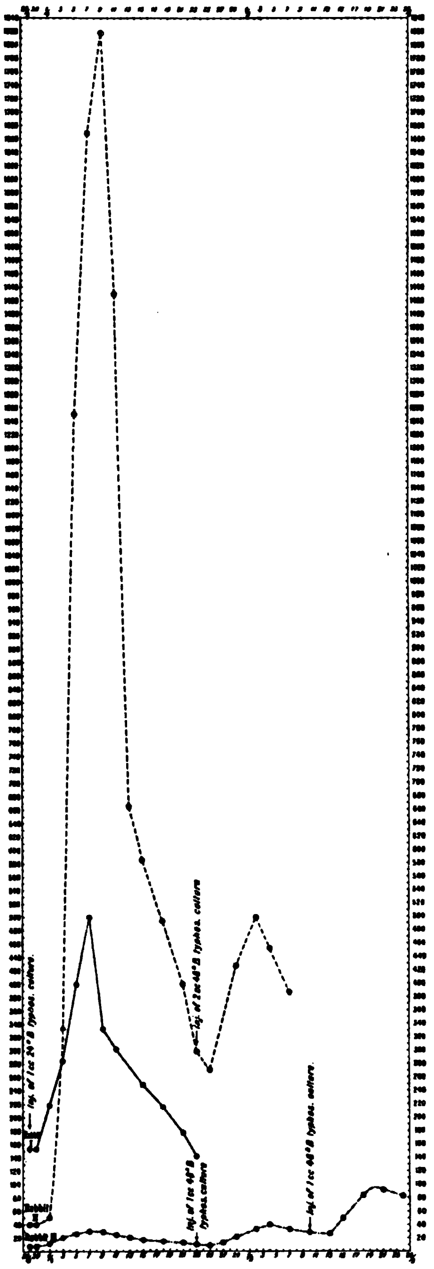
power at the beginning of the experiment was 4. After an injection of 40 cc. 48h culture of Vb. chol., a curve was noticed quite similar to the above mentioned. After a very insignificant fall on the first day the climax was rapidly reached on the 8th day, followed by a decrease, which was at first very sudden and afterwards more gradual.

In curve No. 4 is introduced a new species, the rabbit.

During the 5 weeks, preceeding the experiment, the three rabbits I, II and III had been immunised in exactly the same manner at the same time with the same doses of B. typhos. culture. On the $^{29}/_{3}$ the agglutinating power of rabbit I was 154, of rabbit II 40 and of rabbit III 7,7. On the said day 1 cc. 24h B. typhos. culture was injected into each of the animals; the following curves were like the above mentioned so far as a short period without any perceptible change of the agglutinating power was succeeded by a rise, the maximum of which occurred on the 8th—10th day.

A great number of similar experiments, made under corresponding circumstances, have given quite analogous results.

We are here in accordance with other investigators, who have found the highest agglutinating power 10—13 days after the injection of culture. It is for instance stated by Deutsch[1]), that both the preventive and the agglutinating power of guinea-pigs, immunised against B. typhosus, increased from the 4th day after the injection, reached the maximum on the 10th—12th



Curve No. 4

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[1]) **Ladislas Deutsch:** Contribution à l'étude de l'origine des anticorps typhiques. Annales de l'Institut Pasteur 1899.

day and then slowly decreased. Similar observations are made by Durham and Neisser.

The leading features of this agglutinin curves are thus very like those earlier observed for the other antibodies, mentioned in the introduction, and those described by Levin in the following paper in the case of immunisation against B. coli.

The fluctuations during the production of a series of antibodies have now been examined, viz the tetanus, diphtheria and botulismus antitoxin, rennin antitoxin, the hæmolysins, typhoid, cholera and coli agglutinin, and upon different animals, horses, goats, rabbits and guinea-pigs. Among the great variations in the immunisation against these very different toxic matters, it seems possible to pick out a rule with a rather general applicability, viz. that the course of the immunisation has the same type, as was found by Brieger and Ehrlich in their first experiments with tetanus.

It might then be the most practical way to attack the problems involved in the *production of the antibodies* from a thorough study of for instance the agglutinins, which can so readily and accurately be measured in test tubes alone, and from such facts proceed to the study of other antibodies.

The agglutinin curves seem to be most like those observed in the case of diphtheria antitoxin. In the comparison of their details the following is to be noticed. The fall, which is always very pronounced after an injection of diphtheria toxin, is very insignificant in the agglutinin curves and often not at all to be demonstrated. The second phase on the contrary corresponds in the two cases; and the maximum occurs at the same time, about the 9th day after the injection. A few times this was observed as early as the 7th day, some times this was retarded until the 13th day, approaching what is the case in tetanus and botulismus.

The curve had often a very acute angular form indicating that the amount of agglutinin was rapidly increasing during a very short time, for instance a rise was observed from 8 to 16,000, from 40 to 1820 etc., and then the amount fell down again just as rapidly, so that the initial ordinate of the curve was reached again a few weeks after the injection.

Under these investigations *the individual differences* in the animals, which alway occur in the immunity studies, were met with.

An especially striking example is given by the three rabbits, represented on curve No. 4. After quite a uniform treatment during 5 weeks, the agglutinating power of No. I was 154, of No. II 40 and of No. III 8. An equal amount of B. typhos. culture as used before now produced such different fluctuations, that No. I reached 500, No. II 1820 and No. III 30; yet the type of the curve was equal for them all, the maximum being reached nearly the same day. As a sign of the irregularities, met with, it may be noticed, that it was not No. I (the animal which possessed the greatest amount of agglutinin at the beginning of the experiment) but No. II, the curve of which was so much higher than the others.

It might be of some interest to remember these facts under the endeavour to draw the agglutinin producing qualities into the means of distinguishing the different bacilli of the typhoid- coli group.

Two things must be considered. 1) The regular fluctuations of the agglutinin curve, 2) The different individuality of the animals.

During the immunisation of horses against diphtheria toxin was observed[1]), that in repeating the injection of the same dose of toxin on the same animal, smaller and smaller oscillations were obtained. Sometimes we got similar results for the agglutinins, at is seen from curve No. 2, where the second injection of 100 cc. culture produced a much smaller increase of agglutinin than the first injection. On the contrary, curve No. 4 indicates the quite different manner of reaction of the rabbits I and III. While the second injection of 2 cc. culture in No. I produced a much smaller oscillation than the first (1 cc.), the case was found entirely the reverse in No. III.

The above demonstrated course of immunisation concerns animals, which were previously actively immunised, but exactly the same will be the case for quite fresh animals, never treated before. Owing to the faint virulence of our B. typhos. culture it is easily done to introduce comparatively large doses of it in fresh rabbits or goats, and the following agglutinin curve differs in no respects from those observed in actively immunised animals. Sometimes a very considerable quantity of agglutinin may be obtained after a single injection. Curve No. 5 indicates such a case, where a goat after an injection of 20 cc. 24h B. typhos. culture showed an increase of agglutinin from 0 to 16666.

The proceeding hitherto used, viz. the introduction at once of a great amount of culture into the organism is very unlike what is probably the case under an infections disease, for instance typhoid fever. Such circumstances are perhaps a little better reproduced in the following arrangement of the experiment. Instead

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[1]) Salomonsen et Madsen: Recherches sur la marche de l'immunisation active contre la diphtérie. II.
Annales de l'Institut Pasteur 1899.

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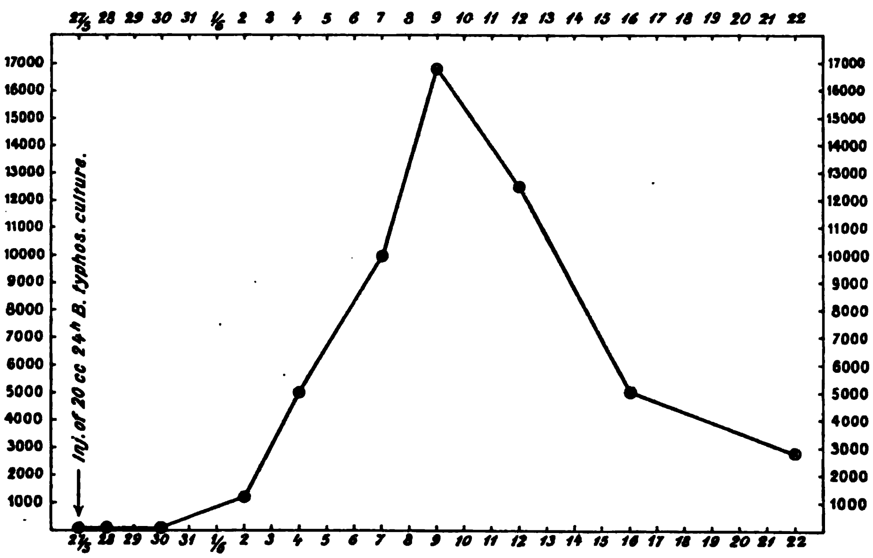

Curve No. 5

of injecting *a single large dose of culture, a comparatively little quantity of a 24h culture was injected daily during a longer period.* Examples are given in the curves 2 and 3.

The goat, referred to in curve 2, showed a slow decrease in agglutinating power during the months, following after the last injection of B. typhos. culture on the $^4/_8$; at about 40 equilibrium occurred. From the $^8/_{12}$ to the $^{31}/_{12}$ 5 cc. of a 24h B. typhos culture was daily injected on the animal, alltogether 120 cc. Its state of health seemed not to be influenced upon under this treatment. During the first 5 days the agglutinin remained in the previous plan of equilibrium, but then increased rapidly to 555 on the $^{27}/_{12}$. From this day a considerable fall occurred in spite of the injections, which were still going on during the 3 following days until the $^{31}/_{12}$.

On curve 3 is given an analogous experiment with Vb. chol. culture, and quite correspondent results were found.

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This experiment is still more striking than the precedent, as the injections were continued 18 days after the maximum was reached. It is of interest to notice the almost critical fall in the 24 hours after the acme, during which the agglutinating power of the serum decreased from 250 to 90. The interpretation of this phenomenon will later on be given.

The last mentioned curve resembles the similar experiments with diphtheria toxin in horses, which were undertaken by Salomonsen and Madsen[1]). This was the case, although these injections, due to practical purposes, were only continued for about 8 days.

PASSIVE IMMUNISATION.

The chief object for our experiments was to examine the fate of the agglutinins, introduced into the circulation. The serum of the animals was previously tested for normal agglutinating power; then the injection of the agglutinin was made, in the case of goats into the jugular vein, in the case of rabbits into the ear vein; the animals were allowed to run around for a few minutes and the first sample of blood after the injection was drawn from the other jugular vein, resp. ear vein.

As examples of the experiments the following may be quoted:

A fresh goat got on the $^{18}/_{4}$ an injection into the jugular vein of 200 cc. cholera-agglutinin from goat. The agglutinating power of the serum, which was 0 before the injection, was immediately thereafter 11,1. As curve No. 6a indicates, the agglutinin fell in the first

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[1]) Recherches sur la marche de l'immunisation active contre la diphtérie. II. Ann. de l'Inst. Pasteur 1899.

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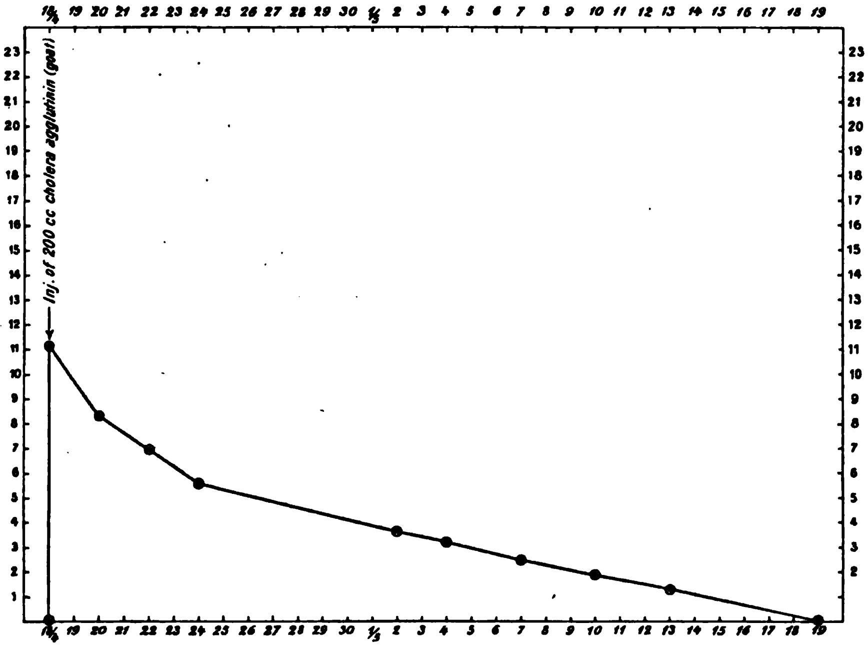
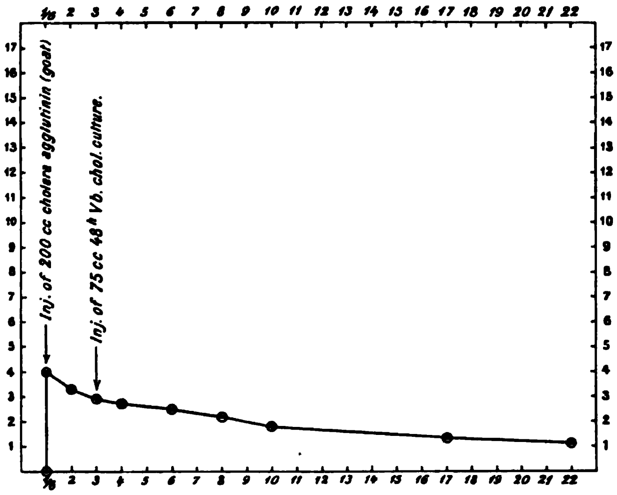


Curve No. 6a



Curve No. 6b

days rapidly, later more gradually and on the $^{19}/_{5}$, one month after the injection, the blood possessed no more agglutinating property.

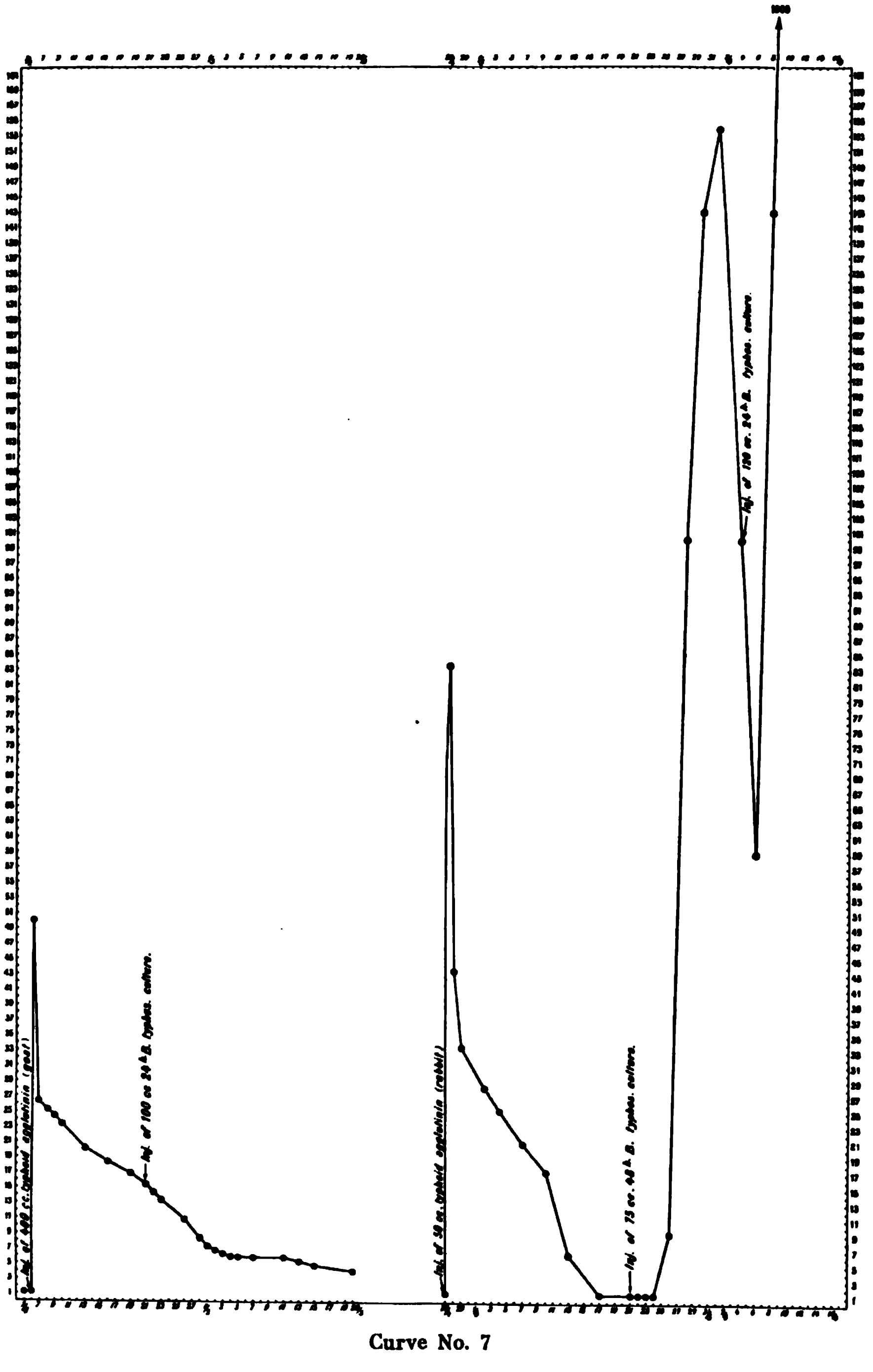
After the injection of 400 cc. typhoid agglutinin (from goat) on another normal goat results quite analogous were obtained (curve No. 7).

It appears from curve 8, that a rabbit, A, after an intravenous injection of 10 cc. typhoid agglutinin (from rabbit) showed a rise in agglutinin from 0 to 62,5. Already on the following day the strenght had fallen down to about the half amount, 36, and the fall was continued during the following days.

Injection of 100 cc. of the same serum on rabbit B increased the agglutinating power of its blood from 0 to 91. The following fall was still more rapid viz. to 37 in the succeeding 24 hours, but got more and more gradual, and one month later, the $^{8}/_{6}$, all agglutinin had disappeared.

It is thus apparent that the general features of the agglutinin curve are the same for goats, injected with goat serum, as for rabbits, injected with rabbit serum. Now it was of interest to see, *if the agglutinins, produced in different species, were identical or not.* To settle this question, the agglutinin curve was examined in animals, on which agglutinin from another species was injected.

Curve No. 9 represents 2 rabbits and 3 cats, which got an injection of 10—12,5 cc. typhoid agglutinin from goat. One rabbit and one cat died the next day under a sudden fall of agglutinin, these animals being not able to stand greater quantities of foreign sera. It appears from the 3 other experiments, that both in the cat and in the rabbit all agglutinin had entirely disappeared within 5—6 days after the injection.


Inj. of 400 cc. typhoid agglutinin (goat)
Inj. of 100 cc 24 h B. typhos. culture.
Inj. of 50 cc. typhoid agglutinin (rabbit)
Inj. of 75 cc. 48 h B. typhos. culture.
Inj. of 100 cc. 24 h B. typhos. culture.
1000

Curve No. 7

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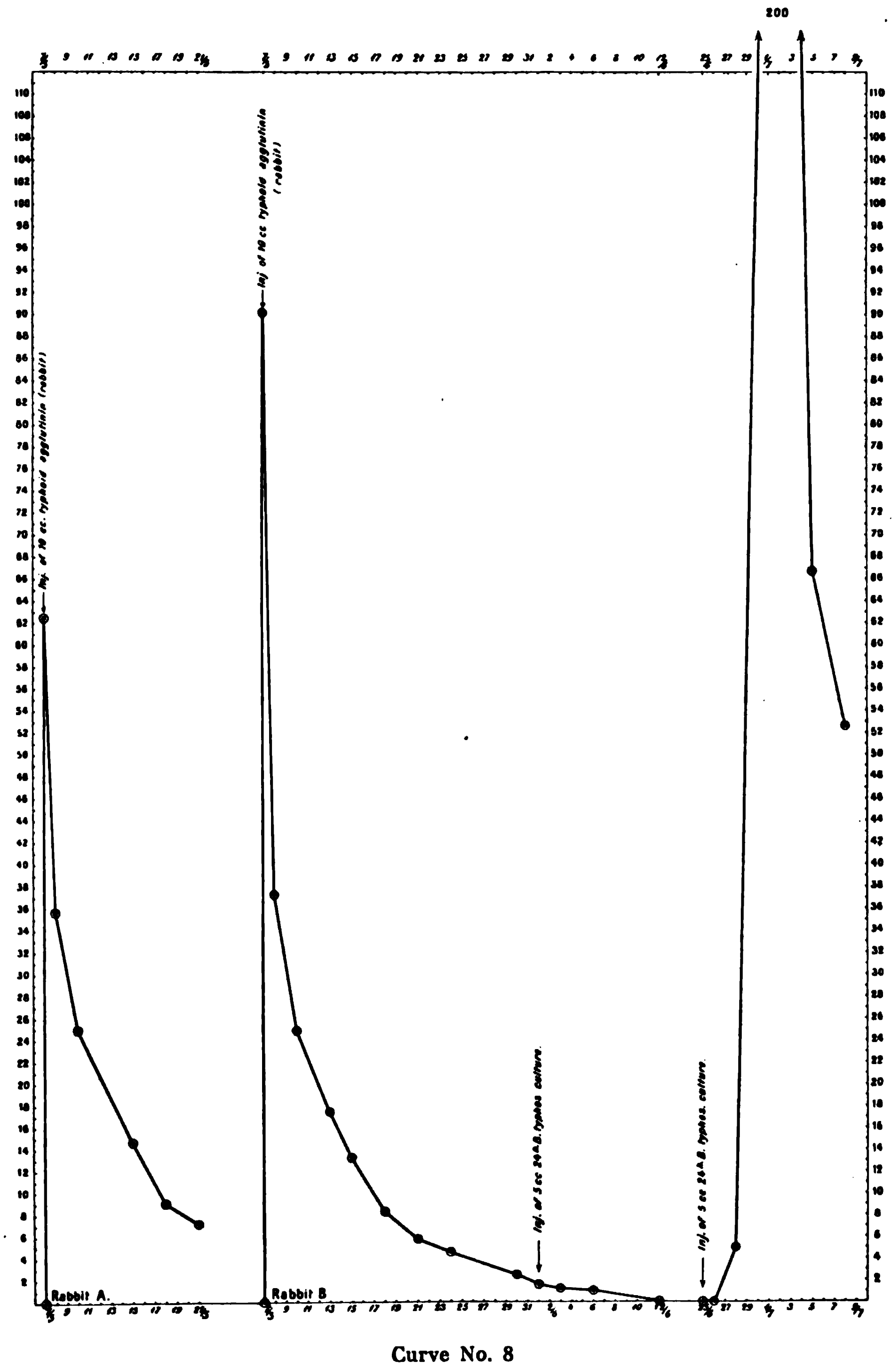
200
Inj. of 10 cc. typhoid agglutinin (rabbit)
Inj. of 10 cc typhoid agglutinin (rabbit)
Inj. of 5 cc 24 h B. typhosus culture.
Inj. of 5 cc 24 h B. typhosus culture.
Rabbit A.
Rabbit B
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
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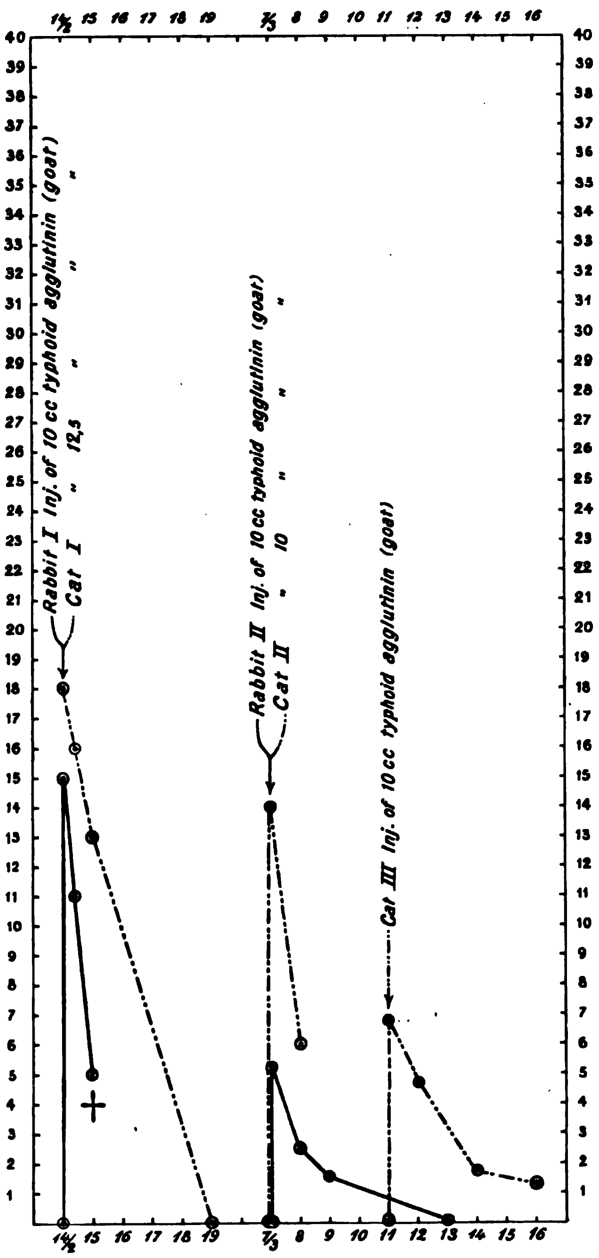
7

Curve No. 8

The quoted experiments suggest really a difference between the different agglutinins so far, as the animals which have got agglutinin from their own species keep it for a comparatively long time, while it disappears in a few days, when the agglutinin originates from another species. To these experiments it can be objected, that a real comparison is very difficult, the agglutinating power obtained after the injections being very different. Therefore in the following experiments it was intended to reach an agglu-



Curve No. 9

tinating power of equal strength immediately after the injection.

Curve No. 10a concerns 4 rabbits of about the same size (2000—2320 gr.). Rabbit α and γ got each 12,5 cc. typhoid agglutinin from rabbit, β and δ each 5 cc. typhoid agglutinin from goat. Preceding experiments had shown, that these 5 cc. goat serum contained exactly the same agglutinating power as the 12.5 cc. rabbit serum, and as it appears from the curve the 4 rabbits from 0 reached about the same point, α 37, γ and δ 33,2, and β 28,5. The rabbits α and γ, which had both got rabbit serum, kept their agglutinin for a comparatively long time 30 and 38 days.

The other two animals β and γ on the contrary, which were injected with goat serum, had already lost their agglutinin on the 9th resp. to the 16th day.

This makes it probable that the typhoid agglutinins from goat and rabbit are different.

From these experiments it seemed at first justifiable to conclude that we had to deal with a law by which it appeared, that the agglutinins of animals of one species always acted as foreign matters, when introduced into the fluids of another species and, as such, at once rapidly disappeared, whereas the agglutinin from an animal of the same species was partly retained for a comparatively long time. As it is well known this view was put forward by Behring some years ago concerning the *antitoxins.*

In the case of agglutinins this supposition must be abandoned in the light of further experimentation. For this served 4 goats. (curve No. 10b). On the $^{19}/_{6}$ was introduced intravenously on goat II 14 cc. typhoid agglutinin (from goat) and on goat I 35 cc. typhoid agglutinin (from rabbit).

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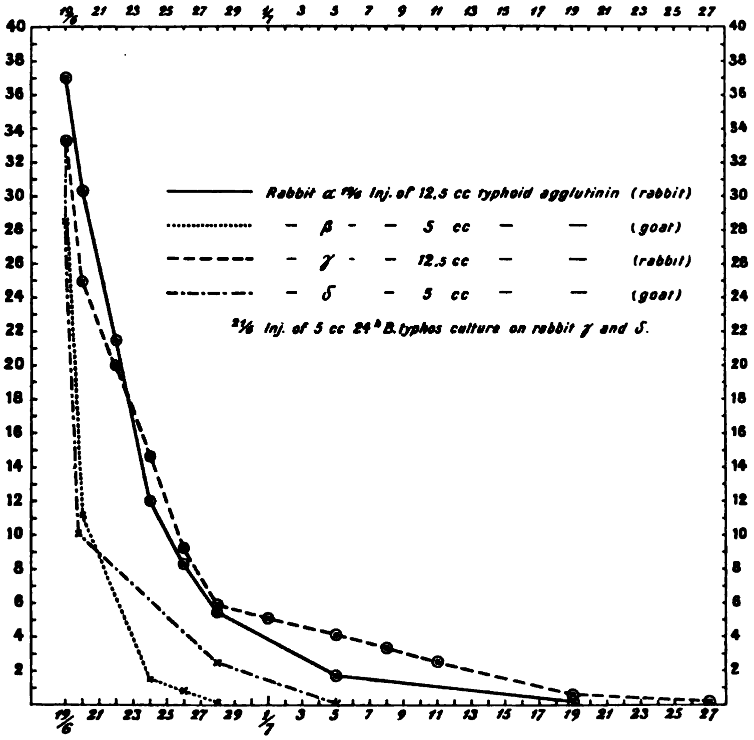
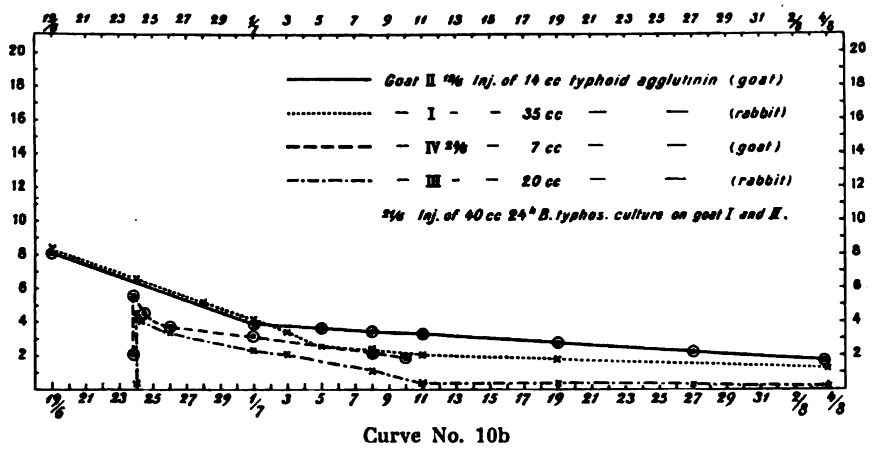


Curve No. 10a



Curve No. 10b

These two doses contained exactly the same amount of agglutinin. On the $^{24}/_{6}$ goat III got 20 cc. of the same rabbit serum and No. IV 7 cc. of the same goat serum. Before the experiment slight traces of agglutinating power could scarcely be demonstrated in the serum of No. I and II, while No. III and IV had a little more, viz. 0,3 and 2. It appears, that after the injection the serum of No. I and II reached exactly the same amount of agglutinin, 8, and that the curves thereafter were running quite parallel during 47 days, when the blood still contained recognizable quantities of agglutinin. As to the other two animals corresponding results were obtained. No. IV died on the 17th day of an accidental infection, when its serum had a strength of 18 and No. III, injected with rabbit serum showed increased agglutinin reaction 14 days after the injection.

The last series of experiments makes it clear, that the fate of the agglutinin from rabbits and goats is quite identical, when introduced into the fluids of goat in corresponding quantities, but that quite other phenomena occur in the organism of rabbits. Thus it is not possible to set up a general law for the fate of the agglutinin of one species within the fluids of another, but each single case requires a special examination.

The disappearance of the agglutinins from the organism, on which they are injected, is very like that of the tetanus- and diphtheria antitoxin, as it is found by several investigators (Behring, Bomstein, Bulloch).

It is not easy to decide if the agglutinins may be considered as *simply diluted in the blood, into which they are introduced.* One of the difficulties is the exact determination of the quantity of blood in the animal

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experimented on. Another is the varying indications for the percentage of serum in the blood.

In the following table is collected those experiments, where the agglutinating power of the serum is examined shortly after the injection. The quantity of blood is judged $^1/_{13}$ of the weight of the animal, and the amount of serum $^2/_3$ of the blood.

Curve No.	Species of animal	Weight in Ko.	Amount of blood in cc.	Amount of serum in cc.	Normal agglutinating power of the blood		Injected serum			Agglutinating power pr cc. after the inject.		The serum injected originated from
								Amount of agglutinin				
					pr. cc.	total	quantity cc.	pr. cc.	total	calcul.	observed.	
7	Goat	32.5	2500	1667	12.5	20838	400	200	80000	48.8	50	Goat (typhoid)
„	„	„	„	„	„	„	50	2500	125000	84.9	83.3	Rabbit „
6	„	22.75	1750	1167	0	0	200	66.67	13333	9.8	11.1	Goat (cholera)
„	„	„	„	„	„	„	200	25	5000	3.7	4	„ „
10a	Rabbit α	2.1	162	108	„	„	12.5	400	5000	41.7	37	Rabbit (typhoid)
„	„ β	2.32	179	119	„	„	5	1000	„	40.8	28.6	Goat „
„	„ γ	2	154	103	„	„	12.5	400	„	43.5	33.3	Rabbit „
„	„ δ	2	„	„	„	„	5	1000	„	46.5	33.3	Goat „
10b	Goat I	18.25	1404	935	Trace	„	35	250	8750	9	8.3	Rabbit „
„	„ II	18.5	1423	849	„	„	14	666.7	9334	9.7	8.3	Goat „
„	„ III	19.5	1500	1000	„	„	20	250	5000	4.9	4.6	Rabbit „
„	„ IV	24.5	1885	1256	2	2513	7	666.7	4667	5.7	5.6	Goat „
9	Rabbit I	2.9	223	149	0	0	10	285.7	2857	17.97	15.2	„ „
„	„ II	3.1	239	159	„	„	10	125	1250	7.4	5.4	„ „
„	Cat II	3.18	245	163	„	„	10	250	2500	14.5	14.3	„ „
„	„ III	4.75	366	244	„	„	20	125	2500	9.5	6.7	„ „

For instance, the weight of the goat mentioned on curve No. 7, was 22,5 Ko., therefore the quantity of blood was estimated to $^1/_{13}$, viz. 2500 cc., and its serum $^2/_3$ of that, viz. 1667 cc. 400 cc. typhoid serum were injected, and thus the vasculatory system contained altogether 2067 cc. of serum. As 1 cc. of the injected serum had an agglutinating power of 200, and the blood having a normal agglutinating power of 12,5 pr. cc. (total 20838), these 2067 cc. of serum contained 100838 units, and one 1 cc. should after the calculation have 48,8, while the experiment gave 50.

It appears from the table, that the calculated values as a rule correspond very well to those observed. That the latter generally are somewhat lower is probably due to the circumstance, that the samples of blood could not always be taken immediately after the injection. This concerns especially the rabbits, where the bleeding always takes some time, in which the rapid decrease of agglutinating power will be noticeable.

The typhoid and cholera agglutinins may thus be considered as simply diluted in the blood of the animal, into which they are injected.

After the separate examination of active and passive immunisation we proceeded to study a *combination of both.*

The first experiment in that way concerned the goat, which is mentioned on curve No. 6. The agglutinating power of the blood, completely disappeared on the $^{19}/_{5}$, was again increased on the $^{1}/_{6}$ by means of an injection of 200 cc. cholera agglutinin from goat. On the $^{3}/_{6}$, when the agglutinating faculty had decreased to 2.8, 75 cc. 48^{h} culture of Vb. choleræ was injected. In a fresh animal this injection had invariably produced the curve, which is typical for the active immunisation; but in this case no change at all took place, and the agglutinin continued a gradual decrease during the following days.

The object of the next experiments was to demonstrate, that the nature of the employed agglutinin had nothing to say in this respect. They were undertaken at the animals mentioned on curve No. 10. The rabbits γ and δ, which had reached exactly the same agglutinating power after injection of respectively rabbit and goat serum, got both on the $^{21}/_{6}$ 5 cc. of a 24^{h} culture of B. typhos. It is evident, that the agglutinating curves were not in the least influenced, but continued in quite the same way as those of the two other rabbits α and β, on which no culture had been injected.

Quite corresponding results were obtained from the experiments on goats (curve No. 10b). Having received the same quantity of rabbit and goat serum the goats I and II showed the same agglutinating power (8). As it appears from the curve the injection of 40 cc. of a 24^{h} culture of typhoid fever bacilli produced not the slightest deviation of the curve.

Amongst the other experiments only the following need to be quoted. On the $^{30}/_{5}$ rabbit B, curve No. 8, had fallen down to 12. On this date 5 cc. 24^{h} B. typhos. culture were injected and produced no reaction at all: the curve continued its fall without interruption. To make sure, that this was not due to a special individual absence of agglutin producing faculty in the rabbit in question, we waited until its serum for about 2 weeks had given no agglutinating reaction at all, and then a new injection of quite the same dose of culture was given. This time was observed a well marked reaction of the same character as that, which was usually found in the case of fresh animals.

Another experiment quite similar to this was undertaken on a goat (curve No. 7). 15 days after an injection of typhoid agglutinin from goat the agglutinating power was reduced to 15; at this time 100 cc. of culture produced no change at all. Some months later on the contrary, the agglutinating power having after another injection of agglutinin again fallen down to the normal titer, 1,2, the injection of 75 cc. of culture was followed by a great reaction. This corresponded to the curve, which is obtained in the case of actively immunised animals. The maximum 153,8 was reached on the 11th day, and when the titer on the $^4/_5$ had fallen down to 100 a new injection of culture was made (120 cc. 24^h). This was followed by a fall, apparently in direct continuation of the preceding, and then a new rise with maximum at 10000 occurred.

Is thus appears, that this animal is not influenced by B. typhos. culture when its serum has an agglutinating power of 15, originating from *introduced* agglutinin.

But after the disapperance of all this, and the blood having only its small normal agglutinating power, a marked reaction is produced by the same factors. This is also the case during active immunisation, the animal itself producing the agglutinin. The contrast between these two conditions appears clearly from the curve.

The *difference between active and passive immunisation* shown by these observations is very distinct, and we are now met with the problem, what is the cause to this difference.

From our observations about the neutralisation in vitro of toxins and antitoxins it is not possible to conclude what is going on in an organism during active immunisation. This was already emphasized by Salomonsen and Madsen[1]) in the case of active immunisation of horses against diphtheria. Take for instance such a horse with 500 antitoxin units pr. cc., on which we inject 100 cc. of diphtheria toxin with L† = 0,2 cc. As these 0,2 cc. in vitro are neutralised by 1 antitoxin unit the 100 cc. toxin injected would in vitro be completely neutralised by 1 cc. of the serum in question. Although this horse contains 50000 times this quantity of antitoxin, yet it shows a very marked antitoxin curve, commencing with a rapid fall, followed by a rise to 1000 units pr. cc.

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[1]) **Salomonsen & Madsen: Recherches sur la marche de l'immunisation active contre la diphtérie.**
Bulletin de l'Acad. Royale des Sciences et des Lettres de Danemark. 1896.

The case is evidently the same as regards the agglutinins.

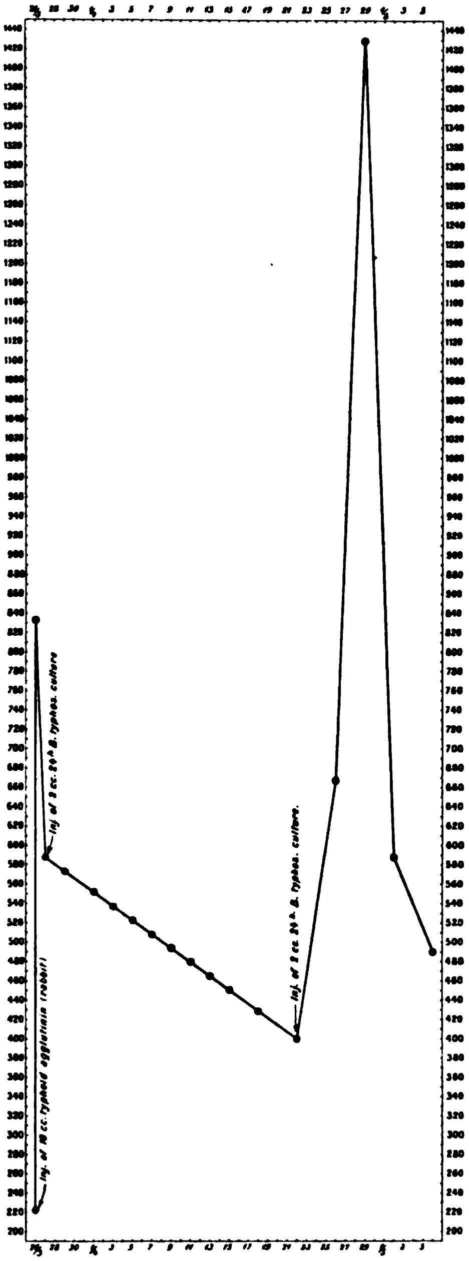
We might ask if the faculty to react with an "agglutinin curve" is not characteristic for the actively immunised organism. That this is not the case appears from the fact above mentioned, that quite fresh animals, never before treated, will give the typical curve after the first injection of culture.

But perhaps an actively immunised organism might have acquired a special strong affinity to the concerned microbes and thus got power to break the combination between the latter and the agglutinin circulating in the blood. This view cannot be maintained as will be seen from following experiments.

When an actively immunised animal is injected with agglutinin, a culture will not produce any agglutinin curve before so long a time has passed, that most of the agglutinin introduced has disappeared (curve Nr. 11.)

This is demonstrated by a rabbit, which from the $^{13}/_{2}$ had been actively immunised against B. typhosus. On the $^{26}/_{3}$, 14 days after the last injection of culture, the agglutinating power of the serum was 222. An injection of 10 cc. strong typhoid agglutinin from rabbit was undertaken, which increased the titer to 833. On the following day it was 588, and the animal then got

VI



Curve No. 11

2 cc. 24h B. typhos. culture introduced intraperitoneally. No agglutinin curve occurred. But 27 days later, when the amount of agglutinin had decreased to 400, the corresponding dose of culture produced a typical curve with a maximum of 1429.

After these observations it seemed of interest to try if the difference between the actively and passively immunised organism should depend upon the circumstance, that the agglutinin in the two cases was not quite identical. It seems that the typhoid and cholera agglutinins (or perhaps rather the antibodies) might possibly possess quite another "neutralising" action upon the microbes in the passively immunised organism and in vitro than in an actively immunised animal.

A number of experiments have been performed with the object of deciding on which point the agglutinin acquires the property to prevent the microbes from producing the typical curve.

The *coagulation* is the most conspicuous phenomenon, which takes place in the agglutinin holding blood after its removal from the organism in which it is produced. To clear up the significance of this phenomenon it was necessary to examine the action of the same agglutinin before and after coagulation. 5 goats were used for the first series of experiments of this kind.

Goat No I got on the $^{8}/_{5}$ 40 cc. B. typhos. culture. The $^{17}/_{5}$ when its serum was supposed to have reached the maximum of agglutinin the following experiments were carried out:

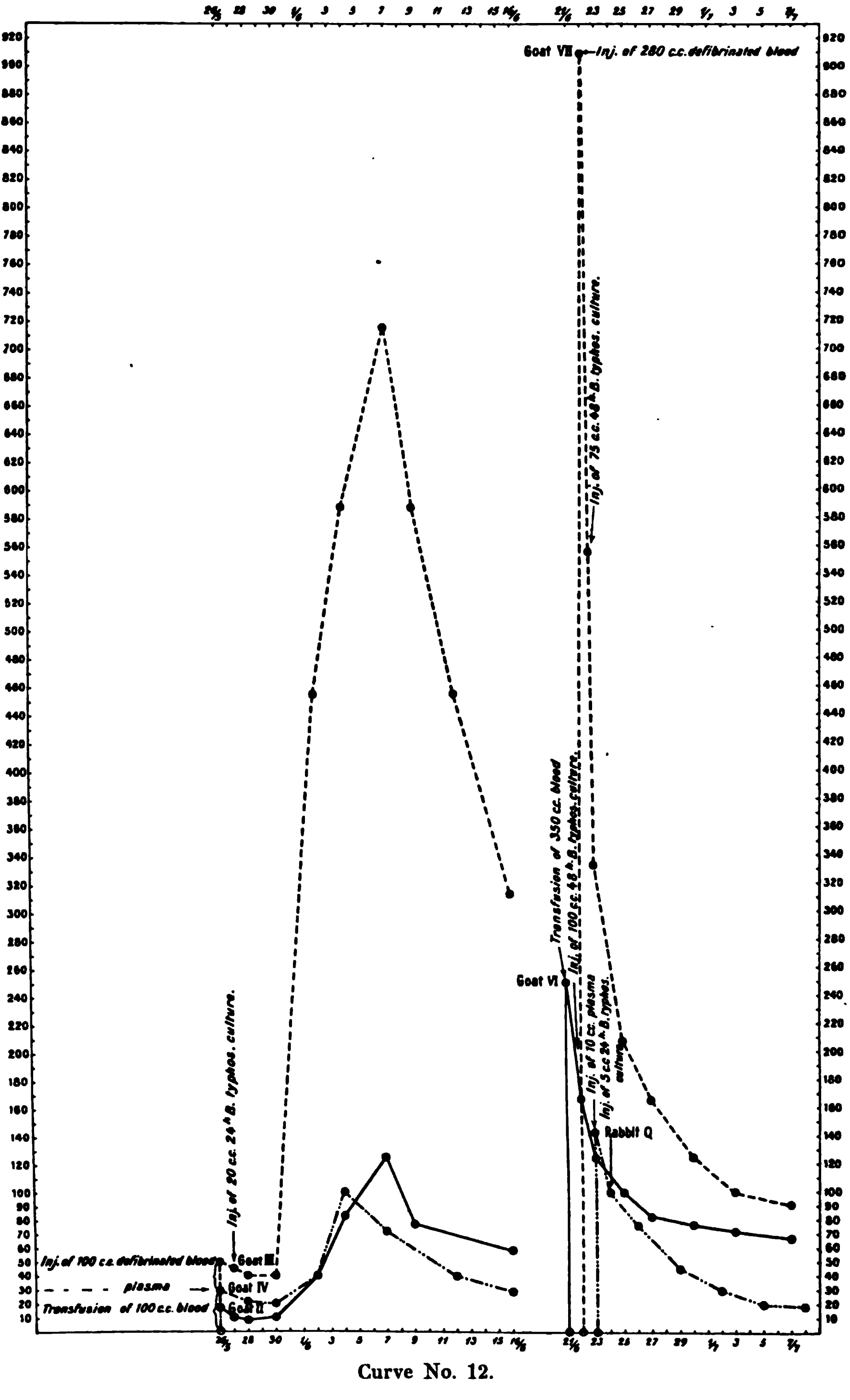
a) From the jugular vein was taken with a syringe 100 cc. of blood, which immediately, and before any coagulation took place, were introduced into the jugular vein of goat II.

b) From the same animal 200 cc. of blood were withdrawn and after defibrination injected intravenously into goat III.

c) 40 cc. infusion of leeches were injected in the jugular vein of No. I. $^{1}/_{2}$ hour later 100 cc. of blood were taken, mixed with 4 cc. infusion of leeches and centrifugalised. The completely clear and fluid plasma was thereafter introduced into the circulation of goat No. IV.

On the next day the animals were getting on all right. Goat II, III, IV and a fresh goat V, which served as control, got an injection of 20 cc. 24^{h} culture of B. typhos. Only a trace of oedema on the place of injection was observed, but no other morbid phenomena. In this respect no difference between the animals could be discovered.

VI



Curve No. 12.

As it appears from curve 12 a typical agglutinin curve took place in all the goats II, III, IV and also in the control animal goat V. (see curve 5). The curve was certainly very different in these four animals, but with our present knowledge to the great individual differences no importance can be placed upon this fact.

This experiment is unsuccessful so far as the animal, which was passively immunised with defibrinated blood also gave a curve, and therefore no conclusion could be drawn from the other experiments with transfusion of blood and plasma. This phenomenon differs quite from all our earlier observations, and the explanation is probably, that there was a too small augmentation in agglutinating power produced in the goats II, III and IV by the injections of agglutinin.

It appears from the curve, that goat No. II after the injection showed 17. The serum of goat No. III, which before the injection possessed a normal agglutinating power of 28,5, increased thereafter to 50, and No. IV reached 29.

This experiment being unsatisfactory, a second was carried out with a considerably greater quantity of agglutinin. This was procured from the actively immunised goats I, II and III, which on the $^{12}/_6$ each got a subcutaneous injection of 100 cc. 24^h B. typhos. culture.

9 days later on the $^{21}/_6$ following experiments were made.

a) From the jugular vein of goat I 350 cc. blood were transferred directly into the jugular vein of a quite fresh goat No. VI. No coagulation at all took place.

b) From the jugular vein of goat II 400 cc. of blood were drawn and defibrinated. After removal of the fibrin by filtration through gauze, the blood was set on a cool place till the following day $^{22}/_6$,

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and then 280 cc. thereof injected intravenously upon another fresh goat No. VII.

c) From the jugular vein of No. III. 400 cc. of blood were drawn and immediately after injected into the same animal (auto-transfusion). To prevent coagulation in the canula and the syringe, these were previously filled with 4 cc. of a very weak infusion of leeches.

On the $^{22}/_6$ 100 cc. 48^h B. typhos. culture were injected on goat No. VI, 24 hours after the transfusion.

On the same day goat VII and III got similar amounts of culture ca 10 hours after the injections.

The result of the experiment was, that goat VI and VII after injection of resp. uncoagulated and defibrinated blood gained a comparatively high agglutinating power. This was resp. 167 and 567 at the moment, when the culture was introduced. No agglutinating curve took place. (see curve No. 12).

A typical one on the contrary occurred in the case of goat No. III, on which an auto-transfusion was undertaken. After another auto-transfusion a quite similar result was obtained.

On curve No. 12 a corresponding experiment with a rabbit is given.

The $^{23}/_6$, 9 days after an injection of 5 cc. B. typhos. culture, 20 cc. of blood was drawn from the jugular vein of a rabbit; as this animal previously had got an injection of 10 cc. of infusion of leeches no coagulation took place. 10 cc. of plasma were easily obtained through centrifugalising and injected intravenously into another rabbit Q. The agglutinating power of the serum was thereby increased to 143. On the following day, when it had fallen down to 100, 5 cc. of a 24^h B. typhos. culture was injected and produced no agglutinin curve.

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In comparing the above experiments the conclusion may be drawn, that the difference in question between an actively and passively immunised animal can not be attributed to a change, occurring in the typhoid-serum through the process of coagulation. This appears from the fact, that the agglutinin producing property of a B. typhos. culture is completely prevented by a *transfusion* of a sufficient amount of agglutininholding blood.

It is also indicated from other facts, that the *agglutinin* as such exits in the organism in which it is produced. A culture of B. typhosus, introduced intravenously into an actively immunised animal will be agglutinated. This is especially striking when a great amount of culture is injected; in this case the clumps of agglutinated bacilli may bring about numerous emboli in different organs.

From the last two series of experiment it is obvious, that the mere presence of introduced agglutinin is not sufficient to prevent an "agglutinin curve", but that the amount of it plays a great role. It seems, that is it not only the absolute amount of agglutinin injected, and not the titer of the serum obtained after this injection, which is of importance. From our small experience we have got the impression, that it is rather the relative increase, which is the main factor.

It is obvious, that this circumstance renders the conclusions drawn from a few experiments very unreliable, and it has made it very difficult for us to continue our experiments in the line, we intended to follow.

We had seen, that a fraction of the blood, transfused into another animal, prevented "agglutinin curve" while a such one arose in the producing animal, containing much more agglutinin.

Our plan then was on a number of actively immunised animals gradually to examine if a culture would produce an agglutinin curve, when such animals previously had got an injection of 1) their own serum, or 2) their own plasma, or 3) their own blood (auto-transfusion). As regards the first two points these problems can only be solved, when the serum, resp. plasma is kept for a comparatively long time. Before the agglutinating power of the animal, experimented on, has decreased to a considerable degree, the serum or plasma injected would in no cases be able to increase the amount of agglutinin to a sufficient extent.

It is a matter of course, that such an increase cannot be obtained in the case of an auto-transfusion, and therefore the experiments of this kind have all given "agglutinin curve". The examination of the two first questions is not yet finished.

The above problems can scarcely be solved by examination of the agglutinating power of the blood alone, the peculiar action of typhoid and cholera serum, which has been the object to these last experiments, probably being due to other antibodies of these sera.

Conclusions.

1) The fluctuations in the agglutinating power of the blood of an actively immunised animal, produced by a single injection of a culture of B. typhos. resp. Vb. choleræ, can be expressed by a regular curve. In this 3 phases can be distinguished:

   1⁰ phase. 3—6 days almost imperceptible decrease of the agglutinating power — or no change.

   2⁰ phase. 3—6 days. An increase with a maximum on the 7th—13th day after the injection (usually 9th-11th day).

3⁰ phase. A decrease, commencing rapidly and gradually passing into

4⁰ phase, where a very slow fall occurs or an equilibrium is reached.

2) A similar curve is obtained in the case of a fresh animal, never treated before.
3) Daily injections of small amount of culture will produce a similar curve. The rise, followed by the fall, will occur *during* the injections.
4) It is probable, that the production of antibodies generally has the type, first described by Brieger and Ehrlich in the case of immunisation against tetanus.
5) Agglutinin, injected into the circulation will disappear rapidly at first and later on more slowly.
6) Agglutinin from goat disappears quickly from the circulation of cat and rabbit, while agglutinin from rabbit keeps for a comparatively long time in the blood of rabbit.

Agglutinin from rabbit and goat, introduced to a similar amount in the circulation ot the goat, will keep for quite the same time.

A difference between agglutinin from goat and rabbit may possibly be supposed.

It is impossible to lay down a general law for the fate of the agglutinin of one species in the fluids of another.

7) The agglutinins may be considered as simply diluted in the blood, into which they are introduced.
8) The introduction of typhoid or cholera serum into an organism (thus passively immunised) will prevent the typical agglutinin curve, which is regularly produced in an actively immunised organism by an injection of culture.

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A fraction of the blood, transfused into another animal, prevents "agglutinin curve", while a such one arises in the producing animal, containing much more agglutinin.

This is a new proof of the fundamental difference between an actively and passively immunised organism.

9) The above mentioned results will apply to both typhoid and cholera agglutinin.

VII.

ERNST LEVIN

COLI AGGLUTININS AND THEIR COURSE OF FORMATION.

The study of the great group of microbes, for which Bacillus typhosus and Bacterium coli are the two predominant types, is getting more and more important.This is of course for a good deal due to their great practical signification, but also a considerable theoretical interest is involved in the solution of the problems connected with them. A great deal of work has been done to penetrate into the characteristics of these organisms and to make a thorough study of every one of their peculiar features. Amongst these their faculty to produce agglutinins has during the last years attracted the greatest attention and has been studied from several points of view.

About the fluctuations in agglutinating power, which are going on into an animal, actively immunised against the said microbes, some information could be found scattered into the papers of a few investigators (Deutsch, Durham, Neisser); but a greater number of experiments in this line were first carried out by Jørgensen and Madsen. In the case of goats and rabbits, which were immunised against B. typhos. and Vb. choleræ it was found, that after an injection of these microorganisms fluctuations in the agglutinating faculty of the blood occurred, which were very like those previously observed during active immunisation against the toxins of tetanus and diphtheria. It was herafter very likely, that a similar rule would apply to the other members of the said group of microbes, and I have therefore, summoned by Dr. Th. Madsen, undertaken a series of experiments in that line.

In this paper only a brief statement of some of my results will be given; more detailed account for the experiments shall later be published.

The chief object for these researches was an investigation of *the phenomena, which occurs into organims, actively immunised against Bact. coli.* I have also tried to examine the influence of different factors upon the course of immunisation, viz. the virulence of the employed culture, and the results of varying modes of immunisation.

The way of proceeding was quite similar that, used of Jørgensen and Madsen in their experiments with typhoid and cholera agglutinin. Different cultures of B. coli were used, both for the immunisation and for the determination of the agglutinin. A 24^h—48^h fresh or boiled culture was injected intraperitoneally on rabbits; this species served for all the experiments. The bloodsamples were drawn from an ear vessel, vein or arteria, and the measurement of the agglutinin was performed by the test tube method, whereafter the results in arbitrary units was indicated in the form of curves.

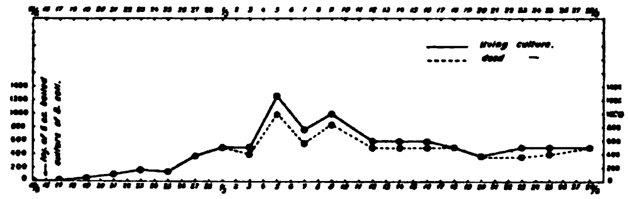
During the experiments a question of certain importance was examined, viz. the differences between dead and living coli cultures, when acted upon by the same amount of agglutinin. For this reason most of the curves are made by means of both kinds of culture, and thus a good material was procured to decide the said question.

Exp. I.

For the purpose of examining the influence of one single injection, 6 cc. of a boiled culture was on the $^{16}/_{2}$ introduced into the peritoneum of a rabbit. Of this culture 0,5 cc. killed rabbits in 8—10 hours, but after the boiling the culture seemed to have lost nearly all toxicity, the rabbit to be examined showing no morbid phenomena at all.

The agglutinating power of the serum, which at the beginning of the experiment was 0, increased after the injection first slowly, thereafter more rapidly and reached the maximum 17 days after the injection, whereafter a gradual decrease occurred.

On curve 1 is represented the fluctuations in the agglutinating power of this rabbit, measured both with living and dead cultures. It appears, that the values obtained by means of the latter, are somewhat lower.

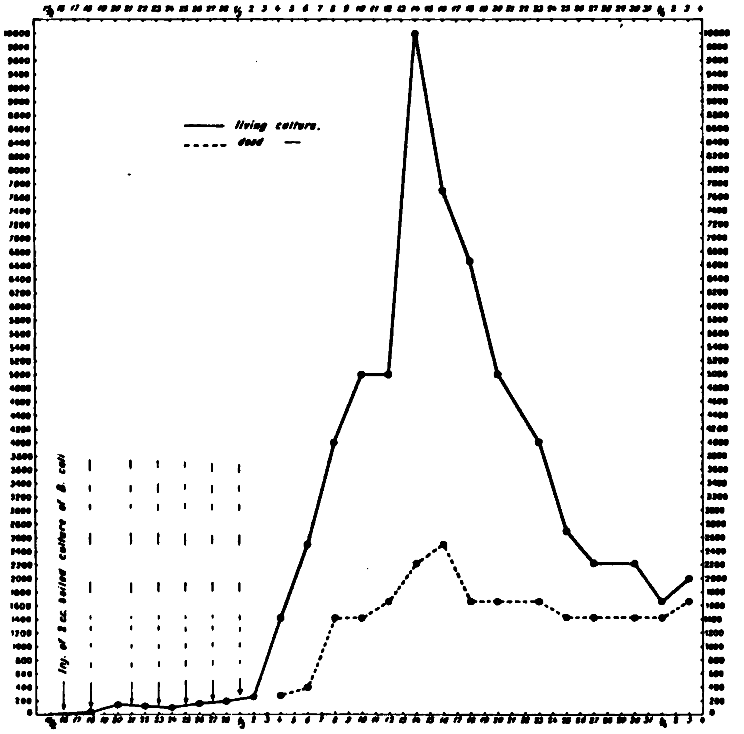


Curve No. 1.

Exp. II.

In this experiment a rabbit was regularly immunised with small doses of dead culture. During a period from the $^{16}/_{2}$ to the $^{1}/_{3}$ 2 cc. of boiled culture was injected every second day. Under this treatment an augmentation in the agglutinating power was observed, this being 0 before the injection took place. During these the increase was comparatively small, but thereafter a considerable rise occurred with a maximum 10,000 on the 13th day after the last injection. (Curve No. 2).

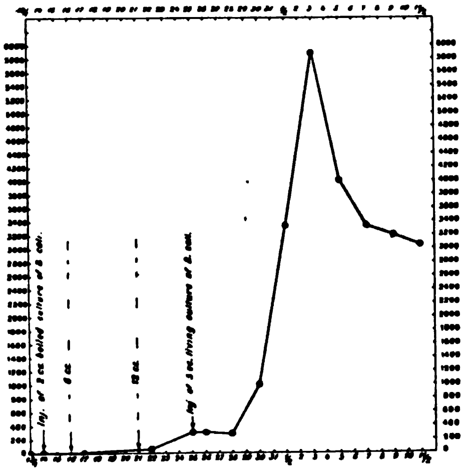
VII



Curve No. 2.

Exp. III

concerns a rabbit, in which the immunisation was commenced with dead and finished with living cultures of an almost avirulent microbe. The result of the experiment was analogous to the previous one with the exception, that the maximum occurred a little earlier,



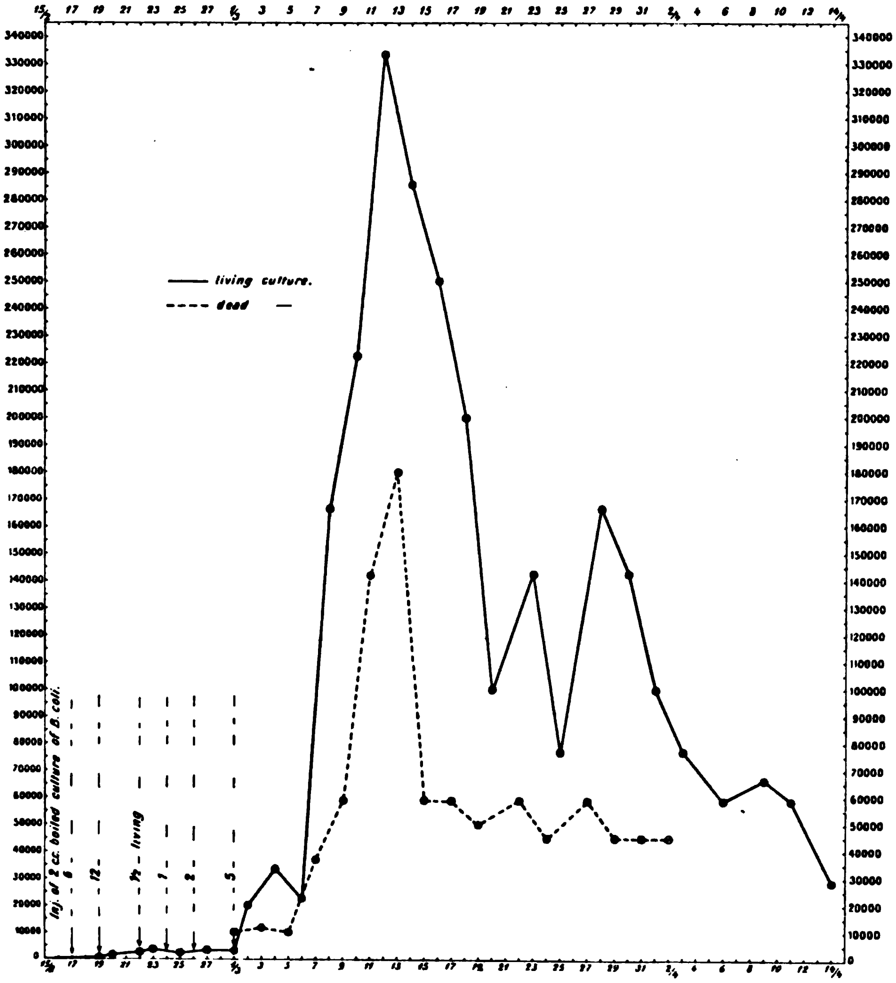
Curve No. 3.

on the 9th day. From curve No. 3. appears all the informations necessary.

The object for

Exp. IV

was to examine the action of an energetic immunisation. This was undertaken during 18 days. In the beginning



Curve No. 4.

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boiled cultures and later on living cultures were employed. The microbe used was the same as in experiment I and II. At last 5 cc. of 24^h culture were injected, 0,5 cc. of which killed control animals in 8—10 hours. The leading features of the following agglutinin curve (No. 4) were quite the same as the above mentioned. It is still to be remarked, that the production of agglutinin in this case was quite extraordinary; the maximum being at 333.333.

In the curve 1, 2 and 4 the agglutinin is determined both with living and dead culture, and a considerable difference may be noted. As a rule the values obtained with dead cultures are only 25—50 $^0/_0$ of the values obtained by the living cultures; this indicates, that by the heating a great deal of the agglutinable matter in the bacteria is destroyed.

It appears from communicated experiments, that the production of agglutinin in rabbits, actively immunised against bacterium coli, follows quite similar laws as those observed in the case of Bacillus typhosus and Vibrio choleræ.

VIII.

THORVALD MADSEN

THE DECREASE OF ANTIBODIES IN THE ORGANISM INDICATED BY A FORMULA.

An antibody introduced into the circulation appears to decrease in a very regular manner. At first, when the amount of antibody is considerable, the fall occurs very rapidly, but gradually it becomes slower and slower. This is clearly seen from the preceding paper[1]) concerning the agglutinins in the circulatory system of different animals.

If we proceed to study the laws of such a phenomenon as a regularly progressing fall, it seemed from the first most perspicuous to figure the amounts as ordinates (x) and the times as abscissæ (t). When we try to find the analytical equation of the curve thus defined, it is natural to expect that there will be a *simple* relation between the velocity of change in the ordinates and the coordinates of the corresponding point.

After different attempts it was found that this relation could be expressed by a differential equation of the following form:

$$-\frac{dx}{dt} \cdot \frac{1}{x^{n+1}} = K$$

n and K being constants, which are characteristic for each case.

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[1]) **Jørgensen and Madsen: The fate of typhoid and cholera agglutinins during active and passive immunisation.**

Integrating this equation we get

$$\frac{-1}{nx^n} = Kt + K_1$$

where K_1 is a new constant.

This equation we put into the following form as being more convenient:

$$\frac{1000^n}{x^n} = ct + c_1 .$$

Taking the left side of this equation as the ordinate, corresponding to t as abscissa, a new curve is obtained, which, is our supposition correct, will be a straight line.

In this straight line

$$X = ct + c_1$$

a series of corresponding values of t and X viz.

$$t_1 \ t_2 \ t_3 \ \ldots\ldots$$
$$X_1 \ X_2 \ X_3 \ \ldots\ldots$$

are to be found.

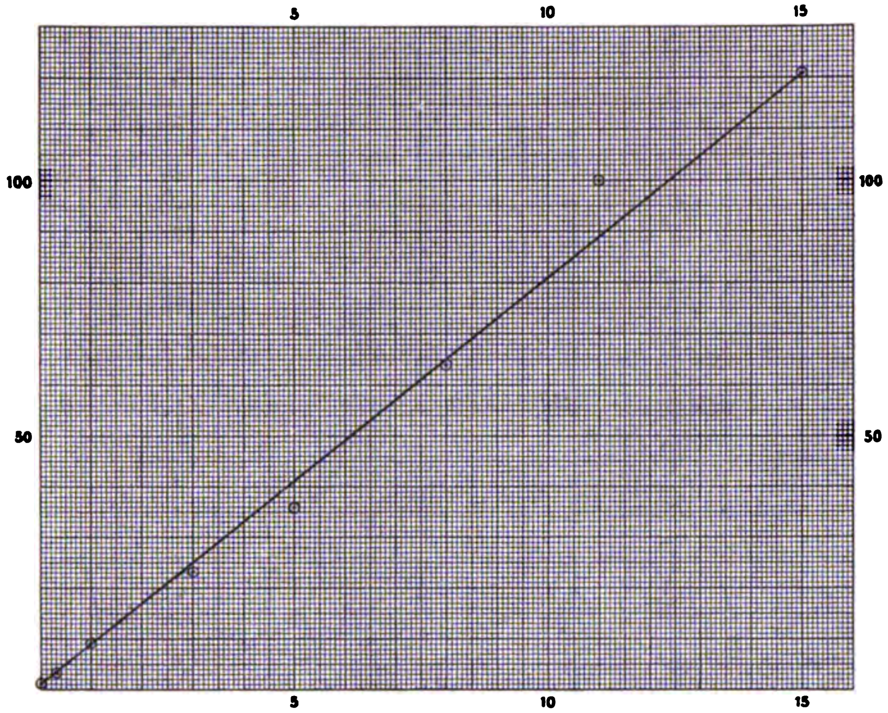
To illustrate the mode of procedure I reproduce the calculations of a special case.

Goat N° VII, curve N° 12 had on the $^{22}/_{6}$ an intravenous injection of typhoid agglutinin from goat; after the injection the blood contained 950 agglutinin units pr. cc. During the following days the agglutinating power of the blood rapidly decreased.

In the table the time t is indicated in days, the amounts of agglutinin observed in arbitrary units:

t	x	X
0	950	1,109
0,3	555	3,246
1	333	9,02
3	207	23,33
5	167	35,86
8	125	64
11	100	100
15	91	120,8

In this case n was found = 2 and for each x the corresponding X was calculated. When the values of X were marked out as ordinates for the different values of t, it was very easy to draw a straight line rather near to the points thus defined.



Having drawn the straight line the constants of its equation could be read directly on the figure, c_1 being the segment of the ordinate axis for $t = 0$ and c being

$$\frac{X_{10} - c_1}{10}$$

In this instance c_1 was found = 1,109
c „ „ = 7,895.

In the following table I have given the different values of x as observed and as calculated from the equation of the straight line.

t	x obs.	in cc.	x calc.	in cc.
0	950	0,0011	949,7	0,0011
0,3	555	0,0018	536,3	0,0019
1	333	0,0030	333,3	0,0030
3	207	0,0048	200,8	0,0050
5	167	0,0060	157	0,0064
8	125	0,0080	124.7	0,0080
11	100	0,0100	106,6	0,0093
15	91	0,0110	91,6	0,0110

The observed values agree very well with those calculated. How satisfactory the correspondence is, appears perhaps better from the figures in the columns besides. These indicate in cc. the doses of serum, of which the resp. agglutinin units are the reciprocal values. It must be remembered that such doses as, for inst., 0,0018 cc. are only interpolated. In this series of test tubes the degree of agglutination used for the test was found to be higher than that of 0,0017 cc. and lover than that of the next tube containing 0,002 cc. Being nearest the first it was judged to correspond to 0,0018 cc.

It was stated above that the value of n par tâtonnement was found = 2. A smaller or greater value of this constant would change the straight line into a curve bending downwards or upwards.

The figures of a few of the experiments mentioned in the preceding paper are given in the following.

Rabbit Q, curve No. 12.

$n = 0,5$
$c_1 = 2,644$
$c = 0,333$

t	x obs.	in cc.	x calc.	in cc.
0	143	0,007	143	0,007
3	100	0 010	75,4	0,013
6	46	0,022	46,4	0,022
9	30	0,033	31,4	0,033
12	20	0,050	22,7	0,044
15	17	0,060	17,1	0,060

Rabbit A, curve No. 8.

$n = 0{,}5$
$c_1 = 1{,}52$
$c = 0{,}158$

t	x obs.	in cc.	x calc.	in cc.
0	625	0,0016	432,9	0,0023
1	357	0,0028	355,1	0,0028
3	250	0,0040	251,4	0,0040
8	147	0,0068	121,9	0,0082
11	91	0,0110	94,2	0,0110
14	72	0,0140	90,4	0,0110

Rabbit B, curve No. 8.

$n = 0{,}5$
$c_1 = 1{,}08$
$c = 0{,}229$

t	x obs.	in cc.	x calc.	in cc.
0	902	0,0011	857,4	0,0012
1	374	0,0027	583,9	0,0017
3	250	0,0040	318,8	0,0032
6	175	0,0057	163,4	0,0060
8	133	0,0075	118,	0,0085
11	83	0,0121	77,2	0,0130
14	58	0,0170	54,5	0,0180
17	46	0,0220	40,4	0,0250
23	25	0,0400	24,8	0,0400

A calculation of the other experiments concerning the decrease of agglutinin in the blood of rabbits and goats shows a similar concordance between the calculated and observed values.

Further it was of interest to determine if the formula would apply to the fate of other antibodies in the circulation of other species. Best examined as regards this point is *diphtheria antitoxin.*

Below is given a calculation of the experiments of

Bomstein[1]), concerning passive immunisation of dogs and guinea-pigs with diphtheria antitoxin.

Dog I.

$n = 0{,}2$
$c_1 = 3{,}2$
$c = 0{,}15$

t	x obs.	x calc.
0	3,0	3,0
4	1,5	1,3
8	0,6	0,6
12	0,3	0,3

Dog II.

$n = 0{,}2$
$c_1 = 3{,}2$
$c = 0{,}19$

t	x obs.	x calc.
0	3,0	3,0
4	1,0	1,0
8	0,5	0,5
12	0,2	0,25

Dog III.

$n = 0{,}2$
$c_1 = 3{,}3$
$c = 0{,}18$

t	x obs.	x calc.
0	2,5	2,5
4	1,0	0,95
8	0,4	0,4
12	0,2	0,2

Guinea-pig.

$n = 0{,}2$
$c_1 = 2{,}316$
$c = 0{,}137$

t	x obs.	x calc.
0	15,0	15
4	6,0	5,2
8	2,5	2,2
12	1,0	1,0
16	0,5	0,53

Bulloch[2]) has examined the durability of passive diphtheria immunity in the case of an ass. A calculation from his figures gives the following result:

$n = 0{,}6$
$c_1 = 14{,}35$
$c = 0{,}2$

t	x obs.	x calc.
0	11,8	16
3	11	11
23	7,5	7,4
47	5,5	5,1
59	4,5	4,3
76	3,2	3,5

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[1]) Bomstein: Zur Frage der passiven Immunität bei Diphtherie. Centralbl. f. Bakt. etc. XXII, 1897, p. 587.

[2]) Bulloch: The durability of passive diphtheria immunity. The Journal of Pathology and Bacteriology. Oct. 1898.

(The values for $t = 99$ and 125 have been omitted as quite uncertain).

By looking at the above calculations it is evident, how satisfactory the concordance is, especially if the unavoidable error of experiment as regards the determination of x is taken in consideration. Also t might not always be exactly determined, the time for the bleeding sometimes being allowed to vary within 6 hours.

Considering this regularity in passive immunisation, it was of interest to examine if the same regularity should be found in the case of *active immunisation.* In the following tables, which refer to the experiments mentioned in the preceding paper, the day, on which the maximum of an agglutinin curve occurs, is taken as 0, and the other values for t are counted from this point.

Goat IV curve No. 12.

$n = 0,5$
$c_1 = 3,162$
$c = 0,2238$

t	x obs.	in cc.	x calc.	in cc.
0	100	0,010	100,0	0,010
3	72	0,013	76,4	0,014
8	40	0.025	40,8	0,025
12	28	0,036	29,2	0,034
18	20	0,050	19,4	0,052

Goat curve No. 1.

$n = 0,3$
$c_1 = 1,331$
$c = 0,0364$

t	x obs.	in cc.	x calc.	in cc.
0	385	0,0026	385,5	0,0026
2	313	0,0032	322,8	0,0031
4	270	0,0037	278,6	0,0036
8	200	0,0050	199,5	0,0050
11	174	0,0057	160,7	0,0062
14	124	0,0080	130,8	0,0076
16	91	0,0110	115,1	0,0087

Goat curve No. III.

a. **11/1, inj. of 40 cc. 24 h Vb. chol. culture**

n = 2,5
c_1 = —130
c = 81,8

t	x obs.	in cc	x calc.	in cc.
0	125	—	?	—
2	100	0,010	99,5	0,010
3	59	0,017	59,6	0,017
4	50	0,020	48,1	0,021
5	42	0,024	41,9	0,024
10	33	0,030	29,2	0,034
13	28	0,036	25,8	0,039
18	20	0,050	22,3	0,045

b. **6/2—13/3, daily inj. of 5 cc. 24 h Vb. chol. culture**

n = 2,5
c_1 = 32
c = 320

t	x obs.	in cc.	x calc.	in cc.
0	250	0,004	250	0,004
1	91	0,011	95,8	0,010
2	77	0,013	74	0,014
3	63	0,016	63,3	0,016
4	56	0,018	56,6	0,018
5	52	0,019	51,6	0,019
6	49	0,020	48,3	0,021
7	46	0,022	45,4	0,022
8	43	0,023	43,1	0,023
10	40	0,025	39,5	0,025
12	38	0,026	36,7	0,027
14	36	0,028	34,5	0,029
16	34	0,029	32,8	0,031
18	32	0,031	31,3	0,032
20	30	0,033	30	0,033
22	28	0,036	28,9	0,035

These four examples refer to the fall (third phase), observed in goats, which were actively immunised in different ways against B. typhos. or Vb. choleræ. The concordance between the calculated and observed values is so exceedingly good, that without doubt the conclusion can be drawn, that the decrease of antibody during active immunisation can be expressed by the same formula, as that applied to passive immunisation.

Accordingly the fate of the agglutinin in an actively immunised organism may be conceived in this way:

After a single injection of culture an incubation period is first observed. Then follows the period during which the agglutinin is brought into the circulation slowly at first, then more rapidly and later on slowly again. The maximum of production occurs 2—3 days before the acme of the curve is reached. The production of antibody, to which the injected culture (or toxin) has given rise, ceases at the time when the curve has reached the acme, or is continued to a small extent for a short time after this point has been reached. The antibody present then decreases accordingly to quite a similar law, as would be the case if it was a foreign antibody, injected into the blood.

It is therefore easy to understand, why a regular immunisation will give much better results, when the injections of toxin are given every third day, (as first pointed out by Dean¹), instead of the day, on which the acme is supposed to occur²). The period of production, on an average 9 days, can approximately be divided into three periods: 3 days of incubation, thereafter 3—4 days to the maximum of production and at last 2—3 days to the end of it. Thus an injection given 3 days after another, will have its maximal production at the time, when the effect of the first has ceased. In this way no interruption in the increase of the antibody will take place. If the injection is given on the day of the acme, 6 days or so will pass before the maximum of production is reached and during those 6 days a considerable loss of antibody incurs.

During the second phase the increase, due to the production of antibody, is counteracted by the decrease according to the law mentioned. This decrease at last predominates. These phenomena might be compared

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1) **George Dean: Problems of diphtheria immunity. Transact. of the Pathol. Soc. of London 51. I. 1900.**

2) **Brieger und Ehrlich: Beiträge zur Kenntniss der Milch immunisierter Thiere. Zeitschr. f. Hyg. XII. 1893.**

Salomonsen og Madsen: Recherches sur la marche de l'immunisation active contre la diphthérie. Ann. de l'Inst. Pasteur 1897.

with those occurring when a body is thrown upwards and then owing to the gravitation, again falls to the ground.

The difference between an actively and passively immunised organism is, that in the latter case, the antibody will decrease regularly to 0, while in the former case the antibody after a certain time usually reaches an equilibrium or continues to decrease very slowly. At this point it seems to be most probable that there is a production of antibody, which covers the loss. It appears from the antitoxin and agglutinin curves, that an actively immunised organism, in every case for a certain time, is liable to keep such an equilibrium, which it tries to restore if disturbed, as for instance by a copious bleeding. That this production is not recognisable during the third phase, might probably depend on its relatively small magnitude.

If the immunisation is produced by daily injections of small amounts of culture, quite a similar curve is observed (see f. inst. curve No. 3). Here the remarkable fact may be noticed, that the fall occurs in spite of the continuing injection of culture. The exact concordance between the observed and calculated values of agglutinin, which is perceived during this fall from its very beginning, indicates that the production has quite ceased, the cells probably being fatigued and no longer capable to react on the injections.

In the case of the goat, curve 3a, a negative constant $c_1 = -130$ is observed. This indicates that our starting point for the time $(t = 0)$ is wrong. Infacta glance at the curve reveals that no observation was made between the $^{19}/_1$ and $^{21}/_1$. The acme would have been observed on the $^{20}/_1$, and this is the real starting point from which the mite t has to be counted, giving a positive value for c_1.

The constants n, c_1, and c probably have a special signification, (species, individuality, character of antibody etc.), but an examination of these factors requires a great and extensive material with uniform values.

As regards the decrease of antibody in the organism it seems most natural to ascribe it to a process of transformation. We know that the decrease of antitoxin cannot be due to an excretion through the secretions (milk, urine, saliva, sweat), the antitoxin curves of these being parallel to that of the blood[1]).

The regularity expressed by the formula mentioned, indicates that a real chemical change of the antibody takes place.

In this respect it deserves to be noticed that the above mentioned equation

$$\frac{dx}{dt} \cdot \frac{1}{x^{n+1}} = K$$

includes the equation for respectively monomolecular and bimolecular reaction, when we take n = 0, resp. n = 1.

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[1]) **Salomonsen et Madsen: Recherches sur la marche de l'immunisation active contre la diphtérie. Ann. de l'Inst. Pasteur 1899.**

De i foranstaaende Afhandlinger III—VIII meddelte Undersøgelser ere for største Delen foretagne med Understøttelse af Carlsbergfondet, som har bevilget Dr. Th. Madsen 1650 Kr. til Anskaffelse af Instrumenter m. m.

IX.

MABEL PUREFOY FITZ GERALD (Oxford)
AND
GEORGES DREYER.

THE UNRELIABILITY OF THE NEUTRAL RED METHOD, AS GENERALLY EMPLOYED, FOR THE DIFFERENTIATION OF B. TYPHOSUS AND B. COLI.

Numerous methods, not one of which can be regarded as absolutely reliable, have been proposed for distinguishing B. typhosus from the closely allied B. coli. Amongst them the use of coloured media holds a prominent position.

The idea of making use of colour change in microbiology originated from Helmholtz[1]) who in 1843 employed a litmus gluten solution to detect putrefaction. He found that before this could be perceived by smell, decoloration of the solution took place.

Buchner[2]) in 1885 introduced coloured media into the bacteriological technique. Since then, their utility has become more and more recognised, not only for purposes of diagnosis, but also for demonstrating certain characteristics of a given micro-organism. Buchner himself used a litmus medium to distinguish Vb. cholerae from similar forms.

Cahen[3]) maintained, that together with the change of colour produced in the medium by the altered reaction, a reduction of the dye itself took place.

Spina[4]) regarded this reduction as a deoxidation because the colour returned if the medium was shaken with atmospheric air.

Razsahegyi[5]) made a large number of experiments with gelatine stained with methylene-blue or methyl-violet for the purpose of differentiating various bacilli.

Noeggerath[6]) showed that by cultivating bacteria on gelatine stained with dyes, the colours of which resembled those of the spectrum, each microbe selected its specific colour.

Behring[7]) found, that by the use of litmus agar, it was possible to distinguish between a weakly and a highly virulent anthrax strain, owing to the greater reducing power of the former.

Kitasato and Weyl[8]) showed, by the use of indigo blue, that certain anaerobes (B. tetanus) were capable of absorbing oxygen.

Notwithstanding the variety of ways in which coloured media have been employed, they have doubtless been of most service in differentiating B. typhosus and B. coli, as will be seen from the following brief summary.

Gasser[9]) found that B. coli in stroke culture on fuchsin agar would decolourise this sooner than B. typhosus.

Würtz[10]) availed himself of a solid medium containing lactose and litmus to differentiate the two bacilli.

Uffelmann[11]) proved by means of methyl-violet gelatine acidified with citric acid that B. typhosus took up the blue colour, so that the colonies were on this account well defined.

Sommaruga[12]) took advantage of different media containing rosolic acid, and regarded the decoloration produced by B. typhosus as due to a reduction, since it occurred even if the medium remained alkaline.

Marpmann[13]) employed malachite-green sulphite agar on which B. coli gives a gray and B. typhosus a green growth, the latter being due to the production of an aldehyde.

Kashida[14]) made use of a 2 % lactose agar containing urea and litmus, in which medium B. coli at first produces a red and subsequently a blue colour, whilst no alteration is caused by B. typhosus.

Rothberger[15]), after experimenting with a large number of colours, recommends neutral red as being far superior to all the others for distinguishing B. typhosus and B. coli. The method employed was to melt the agar and then add two or three drops of a sterilised concentrated solution of neutral red to each test tube. Within 24 hours from the time of inoculation B. coli produced a light yellow colour in the medium, accompanied by fluorescence. Rothberger objected to the use of bouillon as it decolourised spontaneously. In a later paper he recalled this statement and rightly so, adding that this spontaneous decoloration only occurred when a freshly made solution of neutral red was used. He also said that no fluorescence appeared in bouillon, which statement is incorrect.

Rothberger used 4 different strains of B. typhosus and 20 of B. coli and maintains that the reaction is constant for B. coli, but does not occur with B. typhosus, B. Friedlænderi and others.

A number of investigators, relying upon Rothberger's results, have recently employed the neutral red method, and have found it both satisfactory and reliable.

Wolff[16]), endeavouring to explain the cause of the reaction, concludes that it is due to a reduction, since the colour returns by reoxidation, and from this and the fact of the tubes being examined at different periods of time after inoculation, accounts for the diversity of results obtained by different investigators. To overcome the possibility of reoxidation when experimenting with neutral red and B. typhosus and B. coli, he covered the cotton wool corks with liquid paraffin and by this means, obtaining the same result as Rothberger and Scheffler, he regards the method as a good one.

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Scheffler [17]) made a further investigation of Rothberger's results and employed a 0.3—0.5 % glucose agar to which he added 1 % conc. neutral red solution. In this medium B. coli produced a decoloration and a well marked fluorescence, which Scheffler regarded as of great importance. He came to the conclusion that a true B. coli gave a reliable reaction and therefore the method could be used to distinguish this bacillus from B. Typhosus in water and fæces etc.

In accordance with Rothberger and Wolff he found that the reaction could also be obtained with certain putrefactive bacteria, so that it was not absolutely specific.

Hunter [18]) who for some time had applied the method to many different strains of B. coli and typhosus says, that although the reaction constantly succeeds with bouillon, the best results are obtained with a shake or stab culture in glucose agar (0.3 to 2 % glucose). He states that the fluorescence is but feebly developed in bouillon, which is not in accordance with the results obtained by us, as a well marked fluorescence was frequently seen in the bouillon cultures. Hunter's final conclusions may be briefly given as follows:

"1) That B. coli communis possesses to a marked degree the power of reducing neutral red, producing a superb canary yellow fluorescent colour of the medium. 2) That the B. typhosus never possesses this power of reduction. 3) That the common pathogenic micro-organisms do not give this reaction. 4) That by means of neutral red it is possible within from 12 to 24 hours to diagnose with absolute accuracy the presence of B. coli communis"; and he finally states that as far as his experience went: "it was possible to diagnose by means of neutral red the true coli group from the typhoid group of micro-organisms."

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Quite recently Makgill [19]) and Savage [20]) have used the method for the examination of water. Both authors regard the reaction as a very delicate test for the presence of B. coli and state that a negative result may be relied upon as evidence for the probable absence of the bacillus. Savage agrees with Scheffler, Hunter and Makgill, that the best results are obtained by using glucose agar and recommends $^1/_2$ $^0/_0$ glucose agar to which neutral red has been added.

Rothberger, Hunter and Makgill found B. Friedländeri, B. pyocyaneus etc. unable to reduce neutral red, while B. enteritidis of Gaertner and some anærobic forms (B. tetanus etc.) were able to do so.

Since the publication of the researches of Rothberger, Wolff and Scheffler, we have from time to time used this test amongst others for the differentiation of B. typhosus and B. coli. As a rule the result was as expected, but occasionally the reaction failed to occur, therefore we concluded that this test was probably just as unreliable as all the others, the serum test perhaps excepted. After the appearance, of the paper by Hunter, whose results were quite in accordance with those of Rothberger, we thought it of interest to ascertain why certain strains of B. coli would sometimes produce the reaction and sometimes not, and so possibly make the reaction of Rothberger more reliable. —

The results of these experiments are shortly recorded here by referring to the accompanying tables.

Ordinary peptone bouillon and agar containing 0.5 $^0/_0$ of 1 $^0/_0$ watery neutral red solution were used for the experiments. Thus the quantity of neutral red employed is exactly the same as that which we found proposed later by Savage. This $^0/_0$ was chosen because the stronger solution used by former investigators, appeared to cause greater uncertainty in the results.

Experiments were also made to see, whether the results were influenced by the addition of the neutral red, before or after sterilisation of the media. Variations on this account being so very slight, the first method was adopted as preferable from a technical point of view, the chance of infection being excluded. The media used were titrated with $^1/_{10}$ normal NaOH, phenolphtalein as indicator, and when specified as neutral in the tables, neutral to phenolphtalein is meant. The amount of $^1/_1$ normal NaOH necessary to neutralise 100 Cc. of the different bouillons was ca. 1.2 to 1.4 Cc. By a normal bouillon is meant, a bouillon proved by the fermen-

TABLE I. (Normal bouillon).

	1.5 H_2SO_4		1.25 H_2SO_4		1 H_2SO_4		0.75 H_2SO_4		0.5 H_2	
	Ty.	Co.	Ty.	Co.	Ty.	Co	Ty.	Co.	Ty.	
Colour before inoculation . .	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	ma
„ 1 day after „ . .	magenta	magenta	magenta	magenta	magenta	red	magenta	salmon pink	magenta	
„ 2 days „ „ . .	„	„	„	„	„	orange yellow	magenta red	yellow	magenta red	
„ 3 „ „ „ . .	„	magenta red	„	salmon pink	magenta red	yellow	salmon pink	canary yellow	red	
„ 4 „ „ „ . .	„	red	„	orange yellow	red	„	„	„	„	
„ 5 „ „ „ . .										
„ 6 „ „ „ . .							yellow	„		
„ 7 „ „ „ . .	„	orange red	salmon pink	yellow	orange red	„			orange red	
„ 11 „ „ „ . .	cherry red	orange			orange	„			orange	
„ 12 „ „ „ . .			orange	„			„	„		
„ 16 „ „ „ . .										

tation test to be free from sugar. The figures prefixed to H_2SO_4 and NaOH in the tables signify the quantity in Cc. of $^1/_1$ normal $H_2 So_4$ and of $^1/_1$ normal NaOH added to 100 Cc. of neutral bouillon or agar.

To prevent error from the use of sulphuric acid we have also experimented with organic acids (acetic, citric) and obtained practically identical results.

As it soon became evident that the results depended upon many and varied causes, a systematical analysis was made with 6 typical strains of B. coli and 5 of B. typhosus. The alterations in each strain were identical, according to the different conditions to which they were subjected. Unless otherwise stated in the tables, the same strain has been used throughout the

0.25 H_2SO_4		Neutral		0.25 NaOH		0.5 NaOH		0.75 NaOH		1 NaOH		1.25 NaOH	
Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co	Ty.	Co.
nagenta	magenta	iron red	iron red	gold	gold	gold	gold	gold	gold	gold	gold	gold	gold
nagenta red	canary yellow	orange	canary yellow	salmon pink	canary yellow	orange red	canary yellow	red gold	yellow gold	orange	orange	yellow gold	red gold
orange	„	orange yellow	yellow	orange red	„	„	„	„	„	orange red	yellow	„	„
yellow	„	yellow	„	„	„	orange	„	„	„	„	„	„	„
„	„	„	„	„	„	„	„	orange	„	„	„	„	„
„	„	„	„	„	„								
				„	„								
„	„	„	„			orange yellow	yellow	yellow	orange	yellow	„	yellow	yellow
						„	orange yellow	„	„			„	„
„	„	„	„	orange	„					„	„		
„	„	„	„	„	„								

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research, thus enabling one to compare the different series of experiments.

The experiments were arranged with the purpose of bringing out clearly the following points:

1) The effect of using normal bouillon of varying acidity and alkalinity.

2) The effect of using bouillon with different $^0/_0$ glucose and of varying acidity and alkalinity.

3) The effect of using bouillon with different $^0/_0$ lactose and of varying acidity and alkalinity.

4) Results produced in the same media by different varieties of the typhoid and coli groups, (i. e. such bacilli which, on the whole, resemble either B. typhosus or

TABLE II. ($^1/_2$ % glucose bouillon).

	1.5 H_2SO_4		1.25 H_2SO_4		1. H_2SO_4		0.75 H_2SO_4		0.5 H_2S	
	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	
Colour before inoculation . .	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	ma
„ 1 day after „ . .	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	ma
„ 2 days „ „ . .	„	„	„	„	„	red	„	„	„	r
„ 3 „ „ „ . .	„	„	„	„	„	„	„	„	„	
„ 4 „ „ „ . .	„	„	„	„	„	magenta	„	„	„	ma
„ 5 „ „ „ . .	„	„								
„ 6 „ „ „ . .	„	„					„	„	„	
„ 7 „ „ „ . .			„	„	„	„				
„ 10 „ „ „ . .	„	„								
„ 11 „ „ „ . .					„	„			„	
„ 12 „ „ „ . .	„	„	„	„			„	„		
„ 16 „ „ „ . .			„	„						

B. coli, but may fail to give one or other of the characteristic tests).

5) Results produced in the same media by other micro-organisms. —

From Table I it will be seen that *B. typhosus seems to possess the power of reducing neutral red,* though not to such an extent as B. coli; *the reduction is therefore not specific to the latter bacillus.* From the column neutral it will be seen, that after 24 hours B. typhosus has altered the colour from iron red to orange, while B. coli has changed it to canary yellow. This is certainly a colour difference, but after 2 days this has become more doubtful, as the colours are

0.25 H_2SO_4		Neutral		0.25 Na OH		0.5 Na OH		0.75 Na OH		1 Na OH		1.25 Na OH	
Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co	Ty.	Co.
nagenta	magenta	iron red	iron red	gold	gold	gold	gold	gold	gold	gold	gold	gold	gold
nagenta	yellow	magenta	salmon pink	orange red	yellow	magenta	cherry red	magenta	cherry red	magenta	canary yellow	magenta	red
red	»	»	yellow	orange	»	»	orange yellow	»	orange red	»	»	»	orange red
»	»	»	»	»	»	»	orange red	»	red	»	»	»	orange yellow
orange	»	»	»	yellow	»	»	magenta red	»	magenta red	»	orange	»	orange
»	»			»	»								
»	»	»	»	»	»	»	»	»	»	»	salmon pink	»	»
						»	orange red	»	orange red			»	»
»	»	»	»	»	»					»	»		
»	»			»	»								

respectively orange yellow and yellow, and after the lapse of 3 days the colour of both is yellow and so it remains till at least the 16th day. In several of the other columns similar conditions can be observed, though they are not so well marked, and when the variations in colour are observed through a sufficient number of days, the ultimate shade is the same for B. typhosus and B. coli.

Table I demonstrates moreover the powerful influence, exerted by the quantity of free acid or alkali, not only on the occurrence of a colour change, but especially on the time of its appearance, and the acid present must be reduced to 0.5 $H_2 SO_4$ before B. coli is able to alter the colour to yellow within 24 hours.

That none of the earlier investigators have mentioned or observed the capacity of B. typhosus to reduce neutral red, must be accounted for by their having worked, either with different strains of bacilli or with media not absolutely free from glucose.

From Table II it is seen that the presence of $^1/_2\,^0/_0$ glucose lessens, in fact almost prevents the reduction of the neutral red by B. typhosus; at the same time the reducing power of B. coli is considerably diminished. From column 0.5 $H_2 SO_4$ it will be seen that B. coli is not able to alter the colour at all, and from columns 0.25 $H_2 SO_4$ and neutral, that it has capacity for altering the colour from magenta or iron red to yellow within 1—2 days. Table II also shows that *the quantity of free acid or alkali present, exerts more influence on the results in a glucose medium, than in one that is sugar free.* This is a fact of great importance and since the addition of $^1/_2\,^0/_0$ glucose has been recommended by former experimenters, without any reference being made to the amount of free acid present in the

TABLE III. (1 $^0/_0$ glucose bouillon).

	1.25 H_2SO_4		0.75 H_2SO_4		Neutral		0.75 NaOH	
	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co.
Colour before inoculation	magenta	magenta	magenta	magenta	iron red	iron red	gold	gold
„ 1 day after „	magenta	magenta	magenta	magenta	magenta	salmon pink	magenta	salmon pink
„ 2 days „ „	„	„	„	„	„	red	„	„
„ 3 „ „ „	„	„	„	„	„	magenta red	„	red
„ 4 „ „ „	„	„	„	„	„	„	„	„
„ 6 „ „ „			„	„				
„ 7 „ „ „	„	„			„	„	„	magenta red
„ 12 „ „ „	„	„	„	„	„	„	„	„

medium, the errors, arising from such a factor being overlooked, are possibly very many.

From Tables III, IV, V, VI and VII it is evident, that *with an increase in quantity of glucose to 1, 2, 3, 4 and 5 $^0/_0$ there is a proportional decrease in the power of B. coli to change the colour from magenta to yellow. Therefore under these circumstances, the neutral red reaction cannot be applied to differentiate B. typhosus and B. coli.*

As Rothberger and Scheffler prefer to work with neutral red agar, we have also ascertained what effect the kind and quantity of sugar, degree of acidity and alkalinity etc. had upon the results in this medium.

From Table VIII, in which a shake culture, as proposed by Rothberger, Scheffler and Hunter, has been used, it is to be observed, that it is only in the column neutral, that B. coli has been able to change the colour from red to yellow within 24 hours. In colums 1.5, 1 and 0.5

TABLE IV. (2 % glucose bouillon).

	1.5 H_2SO_4		1.25 H_2SO_4		1 H_2SO_4		0.75 H_2SO_4		0.5 H
	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.
Colour before inoculation . .	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta
„ 1 day after „ . .	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta red	magenta
„ 2 days „ „ . .	„	„	„	„	„	„	„	„	„
„ 3 „ „ „ . .	„	„	„	„	„	„	„	„	„
„ 4 „ „ „ . .	„	„	„	„	„	„	„	magenta	„
„ 5 „ „ „ . .							„	„	
„ 6 „ „ „ . .			„	„	„	„			„
„ 7 „ „ „ . .	„	„			„	„	„	„	„
„ 8 „ „ „ . .					„	„			
„ 10 „ „ „ . .									„
„ 11 „ „ „ . .									
„ 12 „ „ „ . .	„	„	„	„	„	„	„	„	
„ 16 „ „ „ . .							„	„	

TABLE V. (3 % glucose bouillon).

	1 H_2SO_4		Neutral		1 NaOH	
	Ty.	Co.	Ty.	Co.	Ty.	Co.
Colour before inoculation	magenta	magenta	iron red	iron red	gold	gold
„ 1 day after „	magenta	magenta	magenta	magenta	magenta	magenta
„ 2 days „ „	„	„	„	„	„	„
„ 3 „ „ „	„	„	„	„	„	„
„ 4 „ „ „	„	„	„	„	„	„
„ 6 „ „ „	„	„	„	„	„	„
„ 7 „ „ „	„	„	„	„	„	„
„ 8 „ „ „	„	„	„	„	„	„
„ 11 „ „ „	„	„	„	„	„	„

.25 H_2SO_4		Neutral		0.25 Na OH		0.5 Na OH		0.75 Na OH		1 Na OH		1.25 Na OH	
Ty.	Co.	Ty.	Co.	Ty.	Co	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty	Co.
agenta	magenta	iron red	iron red	gold	gold	gold	gold	gold	gold	gold	gold	gold	gold
agenta	red	magenta	red	magenta	cherry red	magenta	cherry red	magenta	red	magenta	red	magenta	red
„	„	„	„	„	„	„	magenta red	„	cherry red	„	„	„	„
„	cherry red	„	„	„	„	„	magenta	„	„	„	„	„	„
„	„	„	„	„	„	„	„	„	magenta red	„	„	„	„
								„	„				
„	„			„	„	„	„			„	magenta		
„	„	„	magenta red	„	„	„	„	„	„	„	„	„	magenta red
										„	„		
„	„			„	„	„	„						
										„	„		
								„	„			„	„
		„	„					„	„				

TABLE VI. (4 % glucose bouillon).

	1 H_2SO_4		Neutral		1 Na OH	
	Ty.	Co.	Ty.	Co.	Ty.	Co.
Colour before inoculation	magenta	magenta	iron red	iron red	gold	gold
„ 1 day after „	magenta	magenta	magenta	magenta	magenta	magenta
„ 2 days „ „	„	„	„	„	„	„
„ 3 „ „ „	„	„	„	„	„	„
„ 4 „ „ „	„	„	„	„	„	„
„ 6 „ „ „	„	„	„	„	„	„
„ 7 „ „ „	„	„	„	„	„	„
„ 8 „ „ „	„	„	„	„	„	„
„ 11 „ „ „	„	„	„	„	„	„

TABLE VII. (5 °/₀ glucose bouillon).

	1 H_2SO_4		Neutral		1 NaOH	
	Ty.	Co.	Ty.	Co.	Ty.	Co.
Colour before inoculation	magenta	magenta	iron red	iron red	gold	gold
„ 1 day after „	magenta	magenta	magenta	magenta	magenta	magenta
„ 2 days „ „	„	„	„	„	„	„
„ 3 „ „ „	„	„	„	„	„	„
„ 4 „ „ „	„	„	„	„	„	„
„ 6 „ „ „	„	„	„	„	„	„
„ 7 „ „ „	„	„	„	„	„	„
„ 8 „ „ „	„	„	„	„	„	„
„ 11 „ „ „	„	„	„	„	„	„

TABLE VIII. (Normal agar. Shake culture).

	1.5 H_2SO_4		1 H_2SO_4		0.5 H
	Ty.	Co.	Ty.	Co.	Ty.
Colour before inoculation	magenta	magenta	magenta	magenta	magenta
„ 1 day after „	cherry red	cherry red	red	red	red
„ 2 days „ „	„	½ yellow ½ red	„	½ canary ½ red	„
„ 3 „ „ „	„	„	„	„	„
„ 4 „ „ „	„	orange	„	„	„
„ 7 „ „ „	„	yellow	„	canary yellow	„
„ 11 „ „ „	red	„	orange red	yellow	orange red

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H_2SO_4 the yellow colour does not appear until after 2 days and then, only in the upper half of the medium. This is a contradiction to the statements of Rothberger and Scheffler, who maintain that the colour change, taking place in the medium, never reaches the upper $^1/_2$ Ctm.

Thus it is obvious, that *the results, obtained in either bouillon or agar, are dependent upon just the same factors,* though in the latter medium the differences are not so striking. This is quite in accordance with our view, that the reaction, if it occurs at all, is best marked in bouillon.

In Table IX, where a stab culture was used, the appearance of the reaction was both slower and less certain than with a shake culture. Therefore the use of the latter is to be preferred if agar is employed as the medium. The results with stab cultures however show

Neutral		0.5 Na OH		1 Na OH		1.5 Na OH	
Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co.
iron red	iron red	gold	gold	gold	gold	gold	gold
red	yellow	orange red	yellow	gold	gold	pale gold	pale gold
„	canary yellow	red	canary yellow	„	„	„	„
„	„	„	„	cherry red	canary yellow	„	„
„	„	„	„	red	„	„	„
„	„	orange red	„	orange red	„	„	„
orange red	yellow	„	yellow	„	yellow	„	„

just the same variations, due to a different degree of acidity etc. etc.

TABLE IX. (Normal agar. Stab culture).

	1.5 H_2SO_4		1 H_2SO_4		0.5 H_2S	
	Ty.	Co.	Ty.	Co.	Ty.	
Colour before inoculation	magenta	magenta	magenta	magenta	magenta	mag
„ 1 day after „	cherry red	cherry red	cherry red	cherry red	cherry red	che r
„ 2 days „ „	„	„	„	„	„	
„ 3 „ „ „	„	orange red	„	„	„	
„ 6 „ „ „	„	orange	„	orange red	„	½ ca
„ 10 „ „ „	„	yellow	orange red	orange	orange red	

TABLE X. ($^1/_2$ % glucose agar. Shake culture).

	1.5 H_2SO_4		1 H_2SO_4		0.5 H_2S	
	Ty.	Co.	Ty.	Co.	Ty.	
Colour before inoculation	magenta	magenta	magenta	magenta	magenta	mag
„ 1 day after „	cherry red	cherry red	cherry red	cherry red	cherry red	che re
„ 2 days „ „	„	½ canary ½ red	„	„	„	
„ 3 „ „ „	„	„	„	„	„	ca yell
„ 4 „ „ „	„	canary yellow	„	orange red	„	
„ 7 „ „ „	„	„	„	canary yellow	„	
„ 11 „ „ „	„	„	„	„	„	

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Table X shows that in agar also, the presence of $^1/_2$ % glucose weakens the power of B. coli to alter the colour

Neutral		0.5 Na OH		1.0 Na OH		1.5 Na OH	
Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty	Co
iron red	iron red	gold	gold	gold	gold	gold	gold
red	red	orange red	red	orange	orange	yellow	yellow
"	$^1/_2$ red $^1/_2$ canary	"	$^1/_2$ red $^1/_2$ canary	"	"	"	"
"	"	"	"	salmon pink	yellow	"	"
"	"	"	"	"	"	"	"
orange red	"	"	yellow	"	"	"	"

Neutral		0.5 Na OH		1 Na OH		1.5 Na OH	
Ty.	Co.	Ty,	Co.	Ty.	Co.	Ty.	Co.
iron red	iron red	gold	gold	gold	gold	gold	gold
cherry red	cherry red	cherry red	cherry red	cherry red	cherry red	cherry red	orange
"	orange red	"	canary yellow	"	canary yellow	"	canary yellow
"	canary yellow	"	"	"	"	"	"
"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"
"	"	"	"	"	"	"	"

from red to yellow, therefore the quantity of free acid present here must also be carefully estimated.

Bouillon is used for all the following experiments, as the comparison of the colours is much easier and their shades are more decided.

Having followed the effect produced by different quantities of acid, alkali, and glucose, which, to a certain extent can also be reduced by B. typhosus, we found it of interest to ascertain what effect the presence

TABLE XI. (1/2 % lactose bouillon).

	1.5 H_2SO_4		1.25 H_2SO_4		1 H_2SO_4		0.75 H_2SO_4		0.5 H_2SO_4	
	Ty.	Co.	Ty.	Co.	Ty	Co.	Ty.	Co.	Ty.	C
Colour before inoculation . .	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	mag
„ 1 day after „ . .	magenta	magenta	magenta	magenta	magenta	cherry red	magenta	magenta	magenta	re
„ 2 days „ „ . .	„	„	„	„	cherry red	orange red	red	red	cherry red	ora yel
„ 3 „ „ „ . .	„	„	„	magenta red	red	„	salmon pink	„	orange red	ora
„ 4 „ „ „ . .	„	magenta red	„	red	orange red	red	orange	„	orange	
„ 5 „ „ „ . .										
„ 6 „ „ „ . .							yellow	„		
„ 7 „ „ „ . .	„	„	magenta red	„	„	„			„	
„ 11 „ „ „ . .	„	„			orange	orange red			„	
„ 12 „ „ „ . .			orange	yellow			„	yellow		
„ 16 „ „ „ . .										

of lactose would have, as this sugar is not reduced by B. typhosus, at least by none of the strains used by us.

From Table XI it is seen that in a $^1/_2$ $^0/_0$ lactose bouillon of different acidity and alkalinity, the power of B. coli to alter the colour is much weakened, (compare Table I, normal bouillon) but at the same time the power of B. typhosus to alter the colour has increased very considerably, so that in the course of a few days the colour of the tubes of B. typhosus and B. coli has become almost the same.

.25 H_2SO_4		Neutral		0.25 Na OH		0.5 Na OH		0.75 Na OH		1 Na OH		1.25 Na OH	
Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co.
agenta	magenta	iron red	iron red	gold	gold	gold	gold	gold	gold	gold	gold	gold	gold
agenta red	canary yellow	salmon pink	red	orange red	canary yellow	cherry red	red	cherry red	red	orange red	salmon pink	cherry red	red
range red	„	„	yellow	„	„	red	orange yellow	red	orange yellow	„	canary yellow	red	orange yellow
„	„	orange yellow	„	„	„	orange yellow	orange	orange yellow	yellow	„	„	orange	canary yellow
range yellow	„	„	„	yellow	„	„	„	„	„	yellow	„	„	„
„	„			„	„								
„	„			„	„								
„	„	„	„	„	„	„	„	„	„	„	„	„	orange
						„	„	„	„			„	„
range	yellow	„	„	„	„					„	„		

TABLE XII. (1 °/₀ lactose bouillon).

	1.25 H_2SO_4		0.75 H_2SO_4		Neutral		0.75 NaOH	
	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co
Colour before inoculation	magenta	magenta	magenta	magenta	iron red	iron red	gold	gold
„ 1 day after „	magenta	magenta	magenta	magenta	salmon pink	orange	orange red	orange
„ 2 days „ „	„	„	red	red	„	„	„	yellow
„ 3 „ „ „	magenta red	red	salmon pink	„	„	„	orange	salmon pink
„ 4 „ „ „	red	„	orange	magenta red	orange	salmon pink	„	„
„ 6 „ „ „			yellow	„				
„ 7 „ „ „	orange red	magenta red			yellow	red	yellow	red
„ 12 „ „ „	yellow	„	„	„	„	„	„	„

TABLE XIII. (2 °/₀ lactose bouillon).

	1.5 H_2SO_4		1.25 H_2SO_4		1 H_2SO_4		0.75 H_2SO_4		0.5 H_2SO	
	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	
Colour before inoculation . .	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	ma
„ 1 day after „ . .	magenta	magenta	magenta	magenta	magenta	red	magenta red	red	magenta	ora re
„ 2 days „ „ . .	„	„	red	red	magenta red	orange red	orange red	orange yellow	cherry red	oran- yell
„ 3 „ „ „ . .	magenta red	red	salmon pink	„	red	red	„	orange red	orange red	ora re
„ 4 „ „ „ . .	red	„	orange	magenta red	orange	„	orange	red	orange	re
„ 5 „ „ „ . .							„	„		
„ 6 „ „ „ . .			yellow	„	orange yellow	magenta	„	„	„	.
„ 7 „ „ „ . .	orange red	magenta red			„	„	„	„	„	.
„ 8 „ „ „ . .					yellow	„				
„ 10 „ „ „ . .									„	.
„ 11 „ „ „ . .					„	„				
„ 12 „ „ „ . .	yellow	„	„	„			„	„		
„ 16 „ „ „ . .							„	„		

Here also the influence of varying acidity and alkalinity is clearly exhibited in the obtained results.

From Tables XII, XIII and XIV, where the quantity of lactose added is respectively 1, 2 and 3 % it will be seen, *that with an increasing quantity of lactose the capacity of B. typhosus to alter the red colour to yellow increases, while the power of B. coli decreases.* This is an important fact. From it arises the possibility of using neutral red as a means for distinguishing B. typhosus from B. coli. The reaction modified in this way, (i. e. 3 % lactose neutral red bouillon) though it cannot be regarded as an infallible test, gives more equable and reliable results than that recommended by the earlier investigators, at all events, this has been the case with those bacilli, which we have had at our disposal.

0.25 H_2SO_4		Neutral		0.25 Na OH		0.5 NaOH		0.75 Na OH		1 NaOH		1.25 Na OH	
Ty.	Co.	Ty.	Co.	Ty	Co.	Ty.	Co.	Ty.	Co	Ty.	Co.	Ty.	Co.
magenta	magenta	iron red	iron red	gold	gold	gold	gold	gold	gold	gold	gold	gold	gold
red	orange red	salmon pink	red	red	orange red	red	orange red	orange	orange red	magenta	orange red	orange	red
„	orange yellow	„	salmon pink	„	„	„	orange	yellow	„	salmon pink	orange	„	salmon pink
orange red	orange red	orange	red	orange red	red	orange red	„	„	red	orange	„	orange yellow	red
orange	„	„	„	orange	„	orange	„	„	„	yellow	salmon pink	„	„
		„	„					„	„				
„	red	„	„	„	„	„	red	„	„	„	„		
„	„	yellow	magenta red	„	„	„	„	„	„	„	„	„	magenta red
										„	„		
„	„			„	„	„	„						
										„	red		
		„	„					„	orange red			„	„

TABLE XIV. (3 % lactose bouillon).

	1.5 H_2SO_4		1.25 H_2SO_4		1 H_2SO_4		0.75 H_2SO_4		0.5 H
	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty
Colour before inoculation . .	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta
„ 1 day after „ . .	magenta	magenta	magenta	magenta red	magenta	magenta red	magenta red	red	magenta
„ 2 days „ „ . .	„	„	„	„	„	red	„	„	-
„ 3 „ „ „ . .					„	orange red			orange red
„ 4 „ „ „ . .	„	„	red	magenta	red	red	orange	magenta	orange
„ 5 „ „ „ . .					orange	„			-
„ 6 „ „ „ . .					orange yellow	magenta red			yellow
„ 7 „ „ „ . .	„	„	orange	„			„	„	
„ 10 „ „ „ . .					yellow	„			-
„ 14 „ „ „ . .	„	„	„	„			„	„	
„ 18 „ „ „ . .	„	„	„	„			„	„	

TABLE XV. (½ % glucose bouillon).

	Neutral							
	Ty.1	Ty.2	Ty.3	Ty.4	Co 1	Co 2	Co.3	Co.4
Colour before inoculation	iron red	iron red	iron red	iron red	iron red	iron red	iron red	iron red
„ 1 day after „	magenta	magenta	magenta red	magenta	magenta	salmon pink	yellow	magenta
„ 2 days „ „	„	„	orange	„	red	yellow	„	red
„ 3 „ „ „	„	„	„	„	„	„	„	magenta red
„ 4 „ „ „	„	„	„	„	„	„	„	magenta
„ 7 „ „ „	„	„	„	„	magenta	„	orange yellow	„
„ 12 „ „ „	„	„	„	„	„	„	orange	„

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5 H_2SO_4		Neutral		0.25 NaOH		0.5 NaOH		0.75 NaOH		1 NaOH		1.25 NaOH	
y.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co.	Ty.	Co.
enta	magenta	iron red	iron red	gold	gold	gold	gold	gold	gold	gold	gold	gold	gold
enta ed	red	magenta	red	cherry red	red	red	orange	cherry red	orange red	salmon pink	red	cherry red	orange
nge ed	orange red	red	"	orange	orange red	salmon pink	"	orange red	orange	"	"	"	"
		orange	magenta red			orange	orange red			orange yellow	magenta red		
nge low	magenta red	orange yellow	"	orange yellow	magenta red	orange yellow	red	orange	orange red	yellow		orange red	"
		yellow	"			yellow	magenta red			"	"		
		"	"			"	"			"	"		
low	"			yellow	"			"	cherry red			orange	cherry red
		"	magenta			"	"			"	"		
"	"			"	"			"	"			"	"
"	"			"	"			"	"			"	"

Thus Table XV shows clearly with what irregularity and dissimilarity 4 different strains of B. coli will reduce the $^1/_2\,^0/_0$ glucose neutral red bouillon as $Co._1$ and $Co._4$ were not able to alter it at all. On the other hand Table XVI shows, that in a normal neutral red bouillon, both of these strains, as well as the other two, change the colour from iron red to a bright canary yellow within 24 hours. At the same time two of the strains of B. typhosus ($Ty._2$ and $Ty._4$) are seen to produce an orange colour. So though a colour contrast may be said to exist, it is not a decided one.

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TABLE XVI. (Normal bouillon).

	Neutral							
	Ty.$_1$	Ty.$_2$	Ty.$_3$	Ty.$_4$	Co.$_1$	Co.$_2$	Co.$_3$	Co.$_4$
Colour before inoculation	iron red	iron red	iron red	iron red	iron red	iron red	iron red	iron red
„ 1 day after „	red	orange	salmon pink	orange	canary yellow	canary yellow	canary yellow	canary yellow
„ 2 days „ „	„	„	„	orange yellow	„	yellow	yellow	yellow
„ 3 „ „ „	orange red	„	orange	yellow	„	„	„	„
„ 4 „ „ „	„	orange yellow	„	„	„	„	„	„
„ 7 „ „ „	orange	yellow	yellow	„	yellow	„	„	„
„ 12 „ „ „	„	„	„	„	„	„	„	„

When 3 $^0/_0$ lactose neutral red bouillon (Table XIV) is used, the reaction takes place in the following way: B. typhosus gradually changes the colour of the medium to yellow, passing through the stages of red, orange red and orange. The ultimate yellow colour is retained for months.

B. coli, on the other hand, changes the colour to red even if the medium is alkaline, and though it may produce decoloration during the first few days in the acid and neutral media, a red colour is gradually regained and an ultimate shade of magenta is obtained and preserved unaltered. The reaction, as modified by us, has the disadvantage, that it does not occur until between the 4th—6th day, otherwise there is much to be gained by its use as:

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1) The results are permanent.

2) It is possible to distinguish certain varieties of the coli and typhoid groups, which cannot be differentiated by the method, as formerly used.

3) The results are not dependent to the same extent upon the degree of acidity and alkalinity of the medium.

Table XIV furnishes proof for the last statement. Though in the results, there are slight variations both in the ultimate shade of colour and the time of its production, the reaction never failed to occur whether the bouillon was strongly acid (1.25 $H_2 So_4$), intensely alkaline (1.25 NaOH) or of intermediate degrees of acidity and alkalinity.

Whatever may have been the colour of the medium before inoculation, whether yellow or red, (i. e. acid, neutral or alkaline reaction) B. typhosus always produced a yellow and B. Coli a magenta or red colour. Thus the colour changes here, are vice versa to those of the old method, where B. coli changed the colour to canary yellow, and B. typhosus left it unaltered, red.

As it is seen from our experiments (Table XVII and XVIII) the reaction, though it does occur in 4 % and 5 % lactose bouillon, is observed with the greatest rapidity and most distinctly in 3 % lactose bouillon. Thus it is evident, that the latter % lactose is the best, and, as far as our experience goes, the only one to be recommended. *A lower % than 3 % should never be used*, as the reaction either does not occur or becomes variable and unreliable as for example in 1 % and 2 % lactose bouillon (Table XII and XIII).

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TABLE XVII. (4 % lactose bouillon).

	$1\ H_2SO_4$		Neutral		$1\ NaOH$	
	Ty.	Co.	Ty.	Co.	Ty.	Co.
Colour before inoculation	magenta	magenta	iron red	iron red	gold	gold
„ 1 day after „	magenta	red	magenta	red	magenta	salmon pink
„ 2 days „ „	„	orange red	red	„	red	yellow
„ 3 „ „ „	„	red	„	salmon pink	salmon pink	salmon pink
„ 4 „ „ „	„	„	„	„	yellow	„
„ 6 „ „ „	orange	magenta	orange red	red	„	red
„ 7 „ „ „	„	„	orange	„	„	„
„ 8 „ „ „	„	„	„	„	„	„
„ 11 „ „ „	yellow	„	„	„	„	„

TABLE XIX. (Normal bouillon).

	$0.5\ H_2SO_4$													
	B. Friedländeri	B. choleræ gallin.	Vb. Metchnikoff	Vb. choleræ	Staph. pyog. alb.	Tetragenus	Sarcina aurant.	B. pyocyaneus	Coccob. prodigiosus	B. diphtheriæ	B. megatherium	B. Friedländeri	B. choleræ gallin.	Vb. Metchnikoff
Colour before inocul.	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	iron red	iron red	iron red
„ 1 day after „	canary yellow	magenta red	magenta red	magenta red	cherry red	cherry red	cherry red	red	cherry red	magenta	magenta	canary yellow	red	orange red
„ 2 days „ „	„	„	„	„	magenta	„	„	orange red	„	„	„	„	„	„
„ 3 „ „ „	„	„	red	„	cherry red	„	„	yellow	„	„	cherry red	„	orange red	orange
„ 5 „ „ „	„	„	orange	„	„	„	„	canary yellow	orange	cherry red	„	„	„	yellow
„ 11 „ „ „	„	„	„	„	„	„	„	„	„	„	„	„	„	„

TABLE XVIII. (5 % lactose bouillon).

	1 H_2SO		Neutral		1 Na OH	
	Ty.	Co.	Ty.	Co.	Ty.	Co.
Colour before inoculation	magenta	magenta	iron red	iron red	gold	gold
„ 1 day after „	magenta	magenta red	magenta	orange red	magenta	orange red
„ 2 days „ „	„	salmon pink	„	„	magenta red	yellow
„ 3 „ „ „	„	„	„	„	red	salmon pink
„ 4 „ „ „	„	red	„	„	salmon pink	„
„ 6 „ „ „	salmon pink	magenta red	red	red	yellow	„
„ 7 „ „ „	„	„	orange	„	„	„
„ 8 „ „ „	orange yellow	„	orange red	„	salmon pink	red
„ 11 „ „ „	„	„	„	„	„	„

Neutral							0.5 Na OH										
Staph. pyog. alb.	Tetragenus	Sarcina aurant.	B. pyocyaneus	Coccob. prodigiosus	B. diphtheriæ	B. megatherium	B. Friedländeri	B. choleræ gallin.	Vb. Metchnikoff	Vb. choleræ	Staph. pyog. alb.	Tetragenus	Sarcina aurant.	B. pyocyaneus	Coccob. prodigiosus	B. diphtheriæ	B. megatherium
iron red	iron red	iron red	iron red	iron red	iron red	iron red	gold	gold	gold	gold	gold	gold	gold	gold	gold	gold	gold
lmon pink	red	red	orange red	red	orange	salmon pink	yellow	yellow	salmon pink	salmon pink	yellow	yellow	yellow	yellow	yellow	magen-ta	salmon pink
range	„	„	„	„	„	„	„	„	„	„	orange	„	„	„	„	„	„
„	„	„	„	orange red	„	„	„	„	„	„	„	„	„	„	„	„	„
„	„	„	yellow	orange	yellow	„	„	„	yellow	„	„	„	„	„	„	„	„
„	„	„	„	„	„	„	„	„	„	„	yellow	„	„	„	„	„	„

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TABLE XX. ($^1/_2$ % glucose bouillon).

	0.5 H_2SO_4													
	B. Friedländeri	B. choleræ gallin.	Vb. Metchnikoff	Vb. choleræ	Staph. pyog. alb.	Tetragenus	Sarcina aurant.	B. pyocyaneus	Coccob. prodigiosus	B. diphtheriæ	B. megatherium	B. Friedländeri	B. choleræ gallin.	Vb. Metchnikoff
Colour before inocul.	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenred	iron red	iron red	iron red
„ 1 day after „	orange red	magenta	magenta	magenta	magenta	cherry red	cherry red	red	red	magenta	magenta	yellow	magenta red	magenta red
„ 2 days „ „	red	„	„	„	„	„	„	orange red	„	„	„	orange red	magenta	magenta
„ 3 „ „ „	cherry red	„	„	„	„	„	„	yellow	„	„	„	cherry red	„	„
„ 5 „ „ „	„	„	„	„	„	magenta red	„	canary yellow	orange red	„	„	„	„	„
„ 8 „ „ „	„	„	„	„	„	„	„	„	„	„	„	„	„	„

When different micro-organisms such as B. Friedlænderi, B. cholerae gallinarum, Vb. Metchnikoff, Vb. cholerae, Staph. pyogenes albus, Tetragenus, Sarcina aurantiaca, B. pyocyaneus, Coccob. prodigiosus, B. diphtheriæ and B. megatherium were cultivated in normal and in $^1/_2$ % glucose neutral red bouillon, (Table XIX and XX) it became evident that in the latter medium *B. Friedlænderi was able within 24 hours to alter the colour from red to canary yellow or yellow,* with the same or even a greater certitude than B. coli. This fact is in direct opposition to the statements of Rothberger, Hunter and Makgill, who all affirm that B. Friedlænderi is not able to produce such a change. From Table XXI it is seen that if 3 % lactose neutral red bouillon is inoculated with the same series of bacilli, B. Friedlænderi and B. pyocyaneus are able to

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eutral							0.5 NaOH										
	Tetragenus	Sarcina aurant.	B. pyocyaneus	Coccob. prodigiosus	B. diphtheriæ	B. megatherium	B. Friedländerl	B. choleræ gallin.	Vb. Metchnikoff	Vb. choleræ	Staph. pyog. alb.	Tetragenus	Sarcina aurant.	B. pyocyaneus	Goccob. prodigiosus	B. diphtheriæ	B. megatherium
n d	iron red	iron red	iron red	iron red	iron red	iron red	gold	gold	gold	gold	gold	gold	gold	gold	gold	gold	gold
en- t	cherry red	cherry red	orange red	red	magenta	magenta	orange	magenta red	magenta red	magenta red	magenta red	cherry red	yellow	yellow	magenta red	magenta	magenta
	„	„	orange	„	„	„	red	„	„	„	magenta	„	„	„	orange red	„	„
	„	„	yellow	orange red	„	„	cherry red	„	„	„	„	„	„	„	„	„	„
	magenta	„	„	„	„	„	„	magenta	magenta	„	„	magenta	cherry red	canary yellow	orange	„	„
„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„	„

alter the colour and produce the yellow shade just as well as B. typhosus. *So that the power of changing the colour in 3 % lactose bouillon is not specific to the latter bacillus.*

Finally, we wished to decide which of the two media ½ % glucose or 3 % lactose neutral red bouillon was the best to employ for distinguishing the bacilli of the typhoid group from those belonging to the coli group. For the purpose of making this point clear, contemporaneous experiments were made with a typical B. coli and B. typhosus and a number of bacilli closely related to each.

These strains have all been carefully studied and the characteristics of each accurately observed by Lector Jensen of the Veterinary College, Copenhagen, who has not only kindly provided us with the above named

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TABLE XXI. (3 % lactose bouillon).

	0.5 H_2SO_4													
	B. Friedländeri	B. choleræ gallin.	Vb. Metchnikoff	Vb. choleræ	Staph. pyog. alb.	Tetragenus	Sarcina aurant.	B. pyocyaneus	Coccob. prodigiosus	B. diphtheriæ	B. megatherium	B. Friedländeri	B. choleræ gallin.	Vb. Metchnikoff
Colour before inocul.	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	iron red	iron red	iron red
„ 1 day after „	orange red	magenta red	magenta red	magenta red	cherry red	cherry red	cherry red	red	magenta red	magenta	magenta	canary yellow	salmon pink	salmon pink
„ 2 days „ „	red	„	„	„	orange red	„	„	orange	„	„	„	yellow	„	„
„ 3 „ „ „	cherry red	„	„	„	„	„	„	canary yellow	magenta	cherry red	„	„	„	cherry red
„ 5 „ „ „	„	magenta	cherry red	„	„	magenta	„	„	„	„	„	„	„	„
„ 8 „ „ „	„	„	„	„	orange	„	„	„	„	„	„	„	„	„

cultures, but has also placed his knowledge entirely at our service. For this and other valuable assistance we tender him our best thanks.

The bacilli examined were 2 varieties of the typhoid group (Sv.18 and 49b) and 3 belonging to the coli group (KE_1, 34, and 51b).

Of the typhoid group neither variety produced fermentation in a lactose medium. Sv.18 produced alkali to the same extent as the B. typhosus used, while 49b was a stronger alkali former.

Each one of the coli varieties fermented lactose. 34 and KE_1 produced acid to a mean degree, but KE_1 at the same time formed a considerable amount of alkali. 51b was a strong acid former.

From Table XXII where a 1/2 % glucose neutral red

Neutral							0.5 Na OH										
	Tetragenus	Sarcina aurant.	B. pyocyaneus	Coccob. prodigiosus	B. diphtheriæ	B. megatherium	B. Friedländeri	B. choleræ gallin.	Vb. Metchnikoff	Vb. choleræ	Staph. pyog. alb.	Tetragenus	Sarcina aurant.	B pyocyaneus	Coccob. prodigiosus	B. diphtheriæ	B. megatherium
n d	iron red	iron red	iron red	iron red	iron red	iron red	gold	gold	gold	gold	gold	gold	gold	gold	gold	gold	gold
rry d	cherry red	cherry red	orange red	magenta red	red	cherry red	salmon pink	salmon pink	salmon pink	red	salmon pink	cherry red	gold	yellow	red	red	salmon pink
	»	»	orange	»	»	magenta	orange red	»	»	»	»	»	»	»	»	orange red	»
gen- a	magenta	»	canary yellow	»	»	»	orange	orange	»	»	»	»	»	»	magenta	»	»
»	»	»	»	magenta	»	»	»	»	cherry red	cherry red	»	magenta	»	»	»	orange	»
»	»	»	»	»	»	»	»	»	»	»	»	»	»	»	»	»	»

TABLE XXII. (¹/₂ % glucose bouillon.)

	1 H_2SO_4							Neutral						
	Ty.	Sv18	49 b	Co.	KE₁	34	51 b	Ty.	Sv18	49 b	Co.	KE₁	34	51 b
Colour before inocul.	magenta	magenta	magenta	magenta	magenta	magenta	magenta	iron red	iron red	iron red	iron red	iron red	iron red	iron red
» 1 day after »	magenta ÷	magenta ÷	magenta ÷	magenta ÷	magenta ÷	magenta ÷	magenta ÷	magenta ÷	red ÷	magenta red ÷	magenta ÷	magenta ÷	magenta ÷	magenta ÷
» 2 days » »	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	orange red ÷	» ÷	magenta ÷	red ÷	red ÷	magenta red ÷	» ÷	orange ÷
» 3 » » »	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	red ÷	» ÷	» ÷	» ÷	magenta red ÷	magenta ÷	» ÷	red ÷
» 4 » » »	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	magenta red ÷	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	magenta red ÷
» 5 » » »	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	magenta ÷	» ÷	» ÷	magenta ÷	magenta ÷	» ÷	» ÷	magenta ÷
» 6 » » »	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷
» 10 » » »	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷	» ÷

bouillon has been used, it will be seen, that it is quite impossible to ascertain to which group, typhoid or coli. the above named bacilli belong. as their power to produce a colour change is under these circumstances nearly the same. The difference in colour produced by the true B. typhosus and B. coli employed for these experiments is also so slight, that it would be quite impossible to distinguish them in this way.

Quite different results are obtained from the same strains of bacilli in 3 $\%_0$ lactose neutral red bouillon (Table XXIII). From column neutral, 0.5 and 1 NaOH it is thus seen that B. typhosus and the 2 bacilli of the typhoid group produce a yellow colour as a final result,

TABLE XXIII. (3 $^0/_0$ lactose bouillon).

	1 H_2SO_4							0.5 H_2SO_4							
	Ty.	Sv18	49 b	Co.	KE$_1$	34	51 b	Ty.	Sv18	49 b	Co	KE$_1$	34	51 b	Ty.
Colour before Inocul.	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	magenta	iron red
" 1 day after "	magenta ÷	magenta ÷	magenta ÷	magenta red ÷	magenta ÷	magenta ÷	magenta red ÷	magenta ÷	magenta ÷	red ÷	red ÷	red ÷	magenta red ÷	orange red ÷	magenta ÷
" 2 days " "	" ÷	" ÷	yellow +	red ÷	magenta red ÷	red ÷	red ÷	" ÷	" ÷	canary yellow +	" ÷	orange red ÷	red ÷	yellow ÷	red +
" 3 " " "	" ÷	" ÷	canary yellow +	orange red ÷	red ÷	" ÷	" ÷	orange red ÷	" ÷	" +	orange red ÷	red ÷	" ÷	orange ÷	orange ÷
" 4 " " "	red ÷	" ÷	" +	" ÷	" ÷	" ÷	" ÷	orange +	" ÷	" +	red ÷	" ÷	" ÷	red ÷	orange yellow +
" 5 " " "	orange red +	" ÷	" +	cherry red ÷	magenta red ÷	magenta red ÷	magenta red ÷	orange yellow +	" ÷	" +	magenta red ÷	magenta red ÷	magenta red ÷	" ÷	yellow +
" 6 " " "	orange yellow +	" ÷	" +	magenta red ÷	" ÷	" ÷	" ÷	yellow +	" ÷	" +	" ÷	" ÷	" ÷	magenta red ÷	" +
" 10 " " "	yellow +	magenta red ÷	" +	" ÷	magenta ÷	" ÷	magenta ÷	" +	magenta red ÷	" +	" ÷	" ÷	" ÷	" ÷	" +

while B. coli and the 3 bacilli of the coli group produce a magenta red colour. In column 0.5 and 1 H_2SO_4 a yellow colour is produced by Ty. and 49b of the typhoid group, but it is seen on the other hand that Sv.18, the second member of the typhoid group, keeps the red colour just in the same way as B. coli and the 3 members of the coli group. Thus we have also here a strong proof of the importance of estimating the quantity of free acid if a neutral red medium is used for differentiating these groups, though it may be of much less consequence in of 3 % lactose than in a ½ % glucose medium.

From Table XXIII it will be seen that the different varieties of the typhoid or coli group, whether they are

eutral					0.5 NaOH							1 NaOH						
9 b	Co.	KE_1	34	51 b	Ty.	Sv18	49 b	Co.	KE_1	34	51 b	Ty.	Sv18	49 b	Co.	KE_1	34	51 b
ron ed	iron red	iron red	iron red	iron red	gold	gold	gold	gold	gold	gold	gold	gold	gold	gold	gold	gold	gold	gold
nary llow	red ÷	orange ÷	red ÷	canary yellow ÷	red +	red +	canary yellow +	orange ÷	orange red ÷	magenta red ÷	yellow ÷	salmon pink +	red +	canary yellow +	red ÷	orange red ÷	red ÷	orange yellow ÷
"	÷ "	÷ "	magenta red ÷	yellow ÷	salmon pink +	orange red +	+ "	÷ "	orange ÷	÷ "	orange red ÷	+ "	orange +	+ "	÷ "	orange ÷	magenta red ÷	.orange ÷
"	÷ "	red ÷	magenta ÷	orange red ÷	orange +	orange yellow +	+ "	orange red ÷	red ÷	magenta ÷	red ÷	orange +	orange yellow +	+ "	magenta red ÷	red ÷	magenta ÷	red ÷
"	magenta red ÷	÷ "	÷ "	red ÷	orange yellow +	yellow +	+ "	red ÷	÷ "	÷ "	magenta red ÷	orange yellow +	yellow +	+ "	÷ "	÷ "	÷ "	magenta red ÷
"	÷ "	magenta red ÷	÷ "	magenta red ÷	yellow +	+ "	+ "	magenta red ÷	magenta red ÷	÷ "	magenta ÷	yellow +	+ "	+ "	÷ "	magenta red ÷	÷ "	magenta ÷
"	÷ "	÷ "	÷ "	÷ "	+ "	+ "	+ "	÷ "	÷ "	÷ "	÷ "	+ "	+ "	+ "	÷ "	÷ "	÷ "	÷ "
"	magenta ÷	÷ "	÷ "	÷ "	+ "	+ "	yellow +	÷ "	÷ "	÷ "	÷ "	+ "	+ "	yellow ÷	magenta ÷	÷ "	÷ "	÷ "

strong, medium or weak acid or alkali formers, will produce the colour change. The latter is not dependent only upon a change of reaction. If this were so, the strong acid former of the coli group would turn the colour red or magenta before the weak acid former, which is not found to be the case, as 51b produced the colour change at a later date than KE_1 in spite of this (KE_1) forming in addition to the acid a considerable amount of alkali; on the other hand it appeared that in the typhoid group the strongest alkali producer was the first to give the colour reaction.

Yet by comparing this result with the changes caused by the coli variety KE_1, which, as before stated, produced alkali to the same extent as B. typhosus, but nevertheless changed the colour to magenta, we cannot regard the reaction as due to the quantity of alkali produced. Still further evidence is supplied by the fact, that although in the final results, a red and a yellov colour of the medium will signify an acid and an alkaline reaction respectively, in the intermediate stages of colour change produced by the typhoid group, a varying shade of red may occur in the originally neutral or alkaline media, but yet the reaction be alkaline. Whereas with the coli group, in the originally neutral and alkaline media the reaction is acid, though the colour in the intermediate stages may be a varying shade of yellow, as shown by 51b. (In the Tables ÷ and + signify acid and alkaline reaction respectively).

It is at present quite impossible to know how to account for the difference in the power possessed by B. typhosus and B. coli to produce colour change in 3 % lactose neutral red bouillon. The experiments we have made in this direction not having yielded sufficiently definite results, we can only say that the occurrence of the reaction seems to depend partly upon a formation of alkali or of acid and partly upon a direct decomposition of the neutral red itself, since as a rule it is not possible to regain the original red or yellow colour either by the addition of acid or alkali or by oxidation.

Conclusions.

(1). The difference in the power of B. typhosus and B. coli to produce the respective colour changes in neutral red is a quantitative and not a qualitative one.

(2). If any reliance at all can be placed upon the neutral red method, as proposed by former investigators the following points must be borne in mind.
  - (a) B. typhosus can within certain limits produce similar colour changes as B. coli.
  - (b) The results obtained must be supplemented with other tests.
  - (c) Glucose free bouillon is preferable to agar. The media must be exactly titrated, as a certain quantity of free acid will prevent the occurrence of the reaction.
  - (d) Media containing $^{1}/_{2}$ % glucose or more, must be avoided.
  - (e) Even under the above conditions, it is not possible to use the method for distinguishing members of the typhoid group from those of the coli group.

(3). For the differentiation of B. typhosus and B. coli by means of neutral red, the best medium to employ is 3 % lactose bouillon to which 0.5 % of 1 % watery neutral red (Grübler) has been added.
Within 4—6 days B. typhosus will produce a decided

yellow colour and B. coli a red colour, the original colour of the medium, due to differences in reaction, being quite immaterial to the final results.

Although this method possesses the following advantages:

(a) The results, in comparison to those of the older method, are dependent, to a minor degree only, upon the quantity of free acid or alkali present in the medium.
(b) When once the typical colours have been obtained, no further alteration takes place.
(c) It is possible to differentiate certain members of the typhoid and coli groups which could not be diagnosed by the former method.

it must be remembered that:

(a) Possibly some strains of B. typhosus and B. coli will not produce the colour change.
(b) Different stocks of neutral red do not seem to be equally reducible.

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X.

VILHELM JENSEN

HISTOGENESIS OF THE NODULES PRODUCED BY SUBCUTANEOUS INJECTION OF SACCHAROMYCES NEOFORMANS SANF.

In works dealing with pathogenetic forms of yeast no mention is made, as far as I know, of the changes that occur in the tissues at the point of inoculation *at the time immediately after the inoculation.* I have now, at the suggestion of Professor Salomonsen, taken this question in hand.

Earlier investigators have waited till the death of the inoculated animals before making their researches, or, if death did not result, they have confined themselves to the examination of the nodules in an advanced stage of development. The fact that these nodules are generally called „tumors“, although it is acknowledged that the greater part of them consist of yeast lying in a mesh of connective tissue, makes it all the more desirable that they should be examined more closely.

Sanfelice gives, in his first paper on Saccharomyces neoformans (Zeitschrift für Hygiene. vol. XXI p. 40), the following account of a microscopical examination of the nodules. „Die Geschwülste . . . lassen ein Netz von jungem Bindegewebe erscheinen in dessen Maschen sich hier einige wenige, dort zahlreiche zellige Elemente finden, welche Lymphkörperchen ähnlich sehen, und deren Kern meist an die Peripheri des Zellkörpers gedrängt ist. Neben diesen Elementen kommen so zahlreiche Parasiten vor, dass man sagen kann, die Geschwulst werde vielmehr durch die parasitären Elemente als durch Gewebe gebildet.

An einigen Stellen sind die Zellenelemente haufenweise neben einander gelagert, und einige haben innerhalb ihres Körpers junge Parasiten eingeschlossen. Die Kerne der Zellenelemente sind meist rund und zeigen keine Andeutung der Fragmentation.

Der grösste Theil der Parasiten liegt frei, und einige von ihnen sind gut gefärbt, andere erscheinen hyalin ohne Spur von Färbung."

Page 47: „In der Haut, welche die Geschwulst des Unterhautbindegewebes bedeckt, und in den unter demselben gelegenen Muskeln konnte ich keine Veränderungen bemerken."

I have myself during the last few years made a great many inoculations into guinea-pigs and rabbits with different pathogenetic yeast forms. These experiments will be published elsewhere. Here, where I intend to deal exclusively with histological changes at the point of inoculation in order to gain insight into the development of the yeast nodules, I shall only call attention to a few of these earlier results: The nodules at the point of inoculation are rarely larger than dates, often smaller; this size is reached after a little more than a fortnight; about three weeks after the inoculation the outer skin died away and pealed off like a scab leaving an open wound with thickened edges and ground; this wound might heal, leaving a rather large flat, hairless scar, that is, if the animal had not died at the inoculation; this happened very seldom.

The weight of the animal remained the same or only increased a little during the first few days until the sixth or seventh day, when it commenced to decrease at the same time as the lymph nodules in the groin increased so much that they could be felt. Then the weight decreased till death, or till the wound healed.

The investigations which I am now going to describe, have been made on in all thirteen guinea-pigs, and follow the changes which took place from 3$^1/_2$ hours after the inoculation up to the 35th day.

The three first guinea-pigs weighed each 500 grammes, the ten last ones 300 grammes. For inoculating I used a 5—7 days old culture of Sacch. neoformans Sanf. on a malt agar surface, suspended in a 0,$_7$ % solution of common salt, in such a quantity that the liquid became of a milky appearance. Of this milky fluid $^1/_2$ ccm. was inoculated under the skin in the centre of the right side of the abdominal walls.

The animals were killed by chloroform at the end of the stated periods, and the whole abdomen was placed, during 24 hours, in a saturated watery sublimate solution; it was then, during 24 hours, washed in running water and was cut into horizontal strips, that were hardened in alcohol of increasing strength and then embedded partly in paraffin, partly in celloidin. The paraffin sections were smoothed out with hot water and mounted on slides by means of albuminous water. On examining the sections it was necessary to use many different staining methods.

A combination of staining with *lithium carmine and Claudius' methyl violet picric acid* [I] proved to be the best method for showing the way in which the yeast cells were embedded in the tissues. The receipt is as follows: lithium carmine 2 min. — hydrochloric alcohol 10–20 min. — 1 % watery solution of methylviolet [6B] $^1/_2$–1 min. — half-saturated watery picric acid $^1/_2$ min. — quick rinsing with absolute alcohol — clove oil for decoloration — xylol — balsam. Between the diffe-

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rent stages of staining the preparation is pressed with filtration paper. The results of this staining are very fine, but it can be used only with mounted paraffin sections, the celloidin being unable to withstand the clove oil treatment.

Moreover, the following stains were used: *Weigert's fibrin staining* [II] and *elastin staining* [III]; *thionin,* which especially when using 1 % acetic acid between the staining and the dehydration with alcohol produces a very fine preparation, *hæmatoxyline* and *van Gieson's stain* [V] or *eosin* [VI]; by way of experiment, as stated below, *Ehrlich's triacid solution* [VII] and *Ehrlich-Biondi-Heidenhain's stain mixture* [VIII].

The normal structure of the abdominal walls of the guinea-pig is principally as follows: the skin is hardly 1 mm. thick. It consists, on the outside, of several layers of epithelial cells the first containing the pigment. The connective tissue of the leather-skin is tight, with relatively few elastic threads and a number of small vessels. Under the skin there is a thin layer of loose connective tissue with only few elastic threads, sometimes with a number of fat cells. This loose layer of connective tissue passes, without any definite division, into a fascia layer of about $^1/_{10}$ mm. in thickness containing a number of elastic threads passing especially from one side to another. The yeast cells were generally found after the inoculation on the surface and in the upper parts of this layer. Underneath there are four layers of muscles, separated by thin layers of connective tissue, the thickest between the first and second layers along with a number of elastic threads mainly running lengthwise. The peritoneum is $^1/_{50}$ mm. thick and its inmost layer is especially rich in elastic threads.

As already stated, the greater part of the inoculated yeast cells were found in the connective tissue under the skin and in the upper layers of the fascia. Here the greater part of them were lying in a heap of about 1 ctm. in width and not quite 1 mm. in thickness; some had spread in a thin layer, most of them descending towards the central line.

Guinea-pig 1 ($3^1/_2$ hours).

At the point of inoculation in guinea-pig 1, which was killed three hours and a half after inoculation, the protuberance produced by the injection, the size of half a hazelnut, was seen still unchanged. On section, there was also found an oedematous thickening of the connective tissue of the leather-skin at the point of inoculation. The yeast cells, lying in a compact heap in the connective tissue, look as if they had been left by filtration on the spot where the point of the syringe was. Among these cells a few threads of connective and elastic tissue may be pointed out.

Round all the veins, whose outlines are clearly traced by means of elastin staining, a great many polynuclear leucocytes are seen; similar ones are seen in considerable numbers inside the vessels. Most of the leucocytes are found on the side turning towards the heap of yeast cells (see the affixed table Fig. 1). Evidently a lively emigration of the polynuclear white blood-corpuscles has already taken place, and some of them have reached the yeast cells. The emigration is greater from the veins of the leather-skin than from those lying between the muscles, though it may also be seen in the uppermost layers of the latter. Thus the greater number of the leucocytes are found on the external side of the yeast heap. On staining the cells by the common methods most of them prove not to be granular. Some of them have a two-lobed nucleus and are filled with granules, which are stained yellow red by van Gieson's method and clearly light red by eosin.

Whereas the absorption of the yeast by these cells is quite an exception, a great many injected Saccharomyces are seen to have been ingested by the polynuclear cells which apparently, at least, are without granules. Often 5—6, sometimes 8—10 even 14 yeast cells are found in one white corpuscle. Whilst the leucocytes at their invasion into the tissue are, on an average, 8 μ in diameter they will, under the phagocytose, swell and reach 10—14 μ.

The cells of the connective tissue are of a normal appearance. On this stage the yeast cells already comport themselves in a remarkable way when treated after Weigert's fibrin staining method. While the contents of a single yeast cell on cover-glass preparations from culture are stained uniformly blue, the greater part of the yeast cells which has remained that short time in the animal tissue is discoloured; only a single, rather large, round, sharply outlined, dark blue granule is seen in the quite regular, round cell. At a weaker discoloration the protoplasm still appears of a light blue, and we may then see that this granule is no nucleus, for in another part of the cell, immediately under the membrane, we find the characteristic lenticular protuberance denoting the room of the nucleus (s. Fig. 5).

This characteristic phenomenon is evidently not due to the sublimate fixation, as it does not appear on cover-glass preparations of yeast cells from cultures previously treated with sublimate.

If stained by Claudius' method the yeast cells are coloured uniformly blue; they appear regularly round and are 4—6 μ in size.

Guinea-pig 2 (6 hours) and 3 (8 hours):

In the sections from guinea-pig 2 (six hours) and 3 (eight hours) similar changes as in 1 are found. However the oedema had somewhat decreased, but is still perceptible as a distension of the connective tissue of the leather-skin. The number of the emigrated white blood-corpuscles found in the neighbourhood of the vessels is not so great; they are moving towards the yeast cells, or have already reached them, having advanced more forward into the heap where they stuff themselves with yeast cells. There are still only very few granular leucocytes. The cells of the connective tissue are unchanged, and yeast cells are affected by stains as in guinea-pig 1.

Guinea-pig 4 (18 hours):

In guinea-pig 4 (eighteen hours) nearly the whole of the yeast heap is seen penetrated and, for the greater part, ingested by the leucocytes. The yeast cells do not all of them retain a regular round form; several of them are angular or starshaped; they are no longer stained in the regular way by Claudius' method.

Now all the yeast fungi in the thin lateral and median edges of the yeast heap are ingested by wandering cells; the oedema has disappeared; and in the vessels the number of leucocytes has diminished.

In a few places in the connective tissue, and exceptionally between the leucocytes that are filled with yeast, round cells may be seen. They have a single, great, oviform, cystoid nucleus, which is somewhat lobular; very little protoplasm which takes a brown stain by van Gieson. I have not been able to find any granules in them. These cells are not seen in the neigh-

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bourhood of the vessels. The greater part of them are found in the fissures of the connective tissue, often several together. Their nuclei resemble those of the fix connective tissue cells between which they lie. Transitory forms and figures, which seem to make it likely that they originate through a direct division of the connective tissue cells, are found in the preparations.

Guinea-pig 5 (24 hours):

In sections from guinea-pig 5 (twenty-four hours) the angular and the starshaped yeast cells are more numerous and the break down of the yeast altogether more manifest. The number of mononuclear cells among the yeast cells has increased, a few unchanged connective tissue corpuscles still being visible.

In several places the mononuclear wandering cells gather in little heaps round the yeast, and in several places the border-lines between the individual cells are indistinguishable. Evidently we have before us the beginning of a giant cell formation (s. Fig. 3). Quite exceptional binucleate cells appear; some of these are still fusiform, but they have distinctly increased in size; others show a contraction of the protoplasm between the nuclei. Only very few karyokinetic figures are seen partly as spirems, partly as single stars.

It was not possible to show any difference between white blood-corpuscles, mononuclear wandering cells and the fixed connective tissue cells with triacid or Ehrlich-Biondi-Heidenhain's mixture. The protoplasm in all of them was stained a faint reddish colour without distinct granular delineation; whereas distinctly reddish granules were seen in the above-mentioned granular cells on using these stains.

Guinea-pig 6 (48 hours):

Whereas till now the yeast cells have shown no signs of development, and most of them seem to have decayed, they begin now, in guinea-pig 6, that is, forty-eight hours after inoculation, to change in a characteristic way. In the following I wil speak of these transformed fungi as „*tissue yeast cells*“ as distinct from the inoculated „*culture yeast cells*“.

It was often pointed out, first by Busse, later by Sanfelice, Curtis, a. o., that the yeast cells in the tissues looked quite different to what they did in culture, and further stained in a different way.

Well, *this transformation thus begins in guinea-pigs about forty-eight hours after inoculation.* Among the original 4—6 μ sized yeast cells with thin walls stained uniformly dark blue a few up to 20 μ sized cystoid, granular, light blue stained cells are seen; in the walls of these cells several layers may be distinguished, the outmost appearing as a thick homogeneous capsule (s. Fig. 6). The first of these tissue yeast cells are seen to be surrounded by the above-mentioned circle of mononuclear wandering cells, and seem to be free. In other places a few appear larger than the culture yeast cells. They stand out clearly on being stained with thionin, which gives them a strong red violet tint. They also take a much deeper colour than the decaying culture yeast cells when stained with neutral red.

It cannot with certainty be decided from the preparations at hand whether these commencing tissue yeast cells were ingested by the colourless corpuscles without succumbing or whether they entirely escaped the phagocytes. Either case might happen. In the former case the yeast cells must necessarily have burst the colourless corspuscles as they are always free.

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The number of mononuclear cells has greatly increased; several of them are fusiform; they may very fitly be called „fibroblasts“.

Guinea-pig 7 (3 days) and 8 (4 days):

In guinea-pigs 7 and 8, which were killed after three and four days respectively, the said changes were still more clearly pronounced. The yeast cell heap was surrounded and permeated by a great many long, fusiform cells. Beside these there were seen, on using van Gieson's stain between the yeast fungi, a few connective tissue threads and, on staining with elastin, a very few elastic threads appeared. The latter looked as if they were not destroyed, wholly preserving their normal appearance.

The number of tissue yeast cells has increased. In many places enlarged cells, which have altered with respect to staining, appear, all stages from culture yeast cells to tissue yeast cells being represented. Moreover forms of gemmation are found in no small number, sometimes quite irregular as moderately long threads. Many of these large cells have a distinct capsule with radial stripes, not seen on the yeast cells in older nodules; these stripes are made more perceptible by fibrin staining, which also sometimes makes a dark coloured ring appear round the capsule. Capsules are not found in all tissue yeast cells.

Guinea-pig 9 (5 days):

Five days after inoculation we meet a fresh stage. A great many tissue yeast cells are found here, most of them having capsules, many being gemmiferous. They are lying in a tissue of fibroblasts. The yeast cells found in the phagocytes now appear as small (2—3 μ), angular, starshaped or half-moon shaped remnants staining very irregularly.

On this stage we find in the yeast heap a great many mononuclear large cells whose protoplasm is filled with a great many small vacuoles. This phenomenon is not easily accounted for. Most likely the vacuolation is due to a poisonous action; but in many cases it may be the result of a phagocytosis which has come to an end. The fact that corpuscules representing all transitory forms up to unmistakable yeast cells are sometimes found in the vacuoles (s. Fig. 7), speaks in favour of this hypothesis.

No less difficult to explain is another observation made on this stage, viz. the position of the yeast cells with regard to the fibroblasts that surround them. In several places, they are lying scattered about, not as before in immediate contact with the latter; for on the sections the small yeast heaps are seen separated from the surrounding fusiform cells by clear zones which, on celloidin sections, are always filled with celloidin, making it likely that in life they were filled with a fluid (s. Fig. 2).

There are no connective tissue threads among the fibroblasts; but a considerable number of mitoses show that the peripheric fibroblasts multiply at a great rate (s. Fig. 4). The cells produced by this division differ from the mononuclear cells mentioned above by having larger, more darkly stained nuclei, and a protoplasm which takes a deeper stain. Now — five days after inoculation —, the lymph nodules in the corresponding groin have become palpable, and their size, on section, is $2^{1}/_{2}$ and 2 mm. respectively. In the larger one, situated nearer to the inoculation point, a number of yeast cells with a pronounced thick capsule are placed near the surface. They are all free. In the

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small lymph nodule no yeast cell is yet to be found. Its swelling seems due to the absorption of soluble toxic secretions or products of decay, whilst the yeast cells are still retained in the former.

The yeast cells easily spread themselves along the lymph passages. This is seen also from the fact that in several places, often rather far off from the point of inoculation, small heaps of yeast fungi are located in the neighbourhood of small arteries, as a rule between these and the corresponding veins. Sometimes the yeast cells form a sheath round the artery, the cells making the impression of lying in a hollow existing beforehand.

Guinea-pig 10 (6 days) and 11 (8 days):

After six and eight days the changes mentioned above continue. The yeast cells multiply; the clear spaces already described grow larger; the culture yeast cells go on decaying, disappearing entirely in some places. Among the fibroblasts a fine mesh of connective tissue threads is found, especially near the edges. Gradually all the yeast cells have become surrounded by a ring of fibroblasts and new connective tissue. In several places this ring sends down runners between the muscles. These runners partly enclose the single muscular fibres, some of which are clearly thinned and seem on the point of disappearing. The lymph nodules have grown a little, but yeast cells are still only found in that one nearest to the point of inoculation. In these two guinea-pigs the milt, the lungs, the liver and the kidneys were subjected to microscopical examination. Neither yeast cells nor morbid changes were found in the tissues.

Guinea-pig 12 (20 days):

In guinea-pig 12, which was killed after twenty days, the changes have grown still more pronounced, the skin, however, has not yet been attacked. On the point of inoculation is found a protuberance whose dimensions, on section, are about $3 \times 1^1/_2$ ctm. In the centre of this nodule appear innumerable yeast cells of very different sizes varying from 5—32 μ placed in a hollow, most likely filled with a fluid, in which a few connective tissue threads may be seen but no elastic threads. Only a very few tissue cells are found, most of which having the characteristic vacuolised protoplasm described above with oval nuclei near the surface. A connective tissue growing firmer towards the outside, is seen round this typical mass of yeast. The central hollow space filled with yeast cells is, no doubt, due to a junction of several smaller hollows, partly in the leather-skin, partly down among the muscles. There are also some small nodules, on the whole similar in structure to the greater ones.

Leucocytic infiltration appears nowhere, not even round the vessels.

In this guinea-pig the lymph nodules are enlarged, about the size of peas. The first is entirely permeated by yeast cells; a smaller number is seen in the second; the third, of the size of a hempseed, is still without any at all. Yeast cells are found in the lungs, the milt and, in small numbers, in the kidneys, but I shall not here go into particulars with regard to the changes which take place in the inner organs of the animals.

Guinea-pig 13 (35 days):

This guinea-pig, which was killed thirty-five days after inoculation, illustrates how the continuous growth

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of the yeast cells has produced an atrophy of the covering skin, while the scab begins to be thrown off. Otherwise the appearance is exactly the same as with guinea-pig 12: great heaps of yeast located in a very loose connective tissue whose meshes look as if they were extended by a fluid. Downwards towards the atrophic abdominal muscles the nodule is limited by a firmer connective tissue capsule.

At the same time as the scab is thrown off, the greater part of the yeast cells will, as a rule, empty themselves as a pulpy mass, and the great loss of substance thus caused will generally heal in the course of a few weeks, the animal increasing in weight and making a complete recovery.

It is very doubtful, however, whether this guinea-pig would have escaped death, as guinea-pigs generally do after subcutaneous inoculation. In this animal, the penetration of the yeast cells into the blood passages through a venous wall was distinctly seen—an occurrence I have observed in this one animal only of the great number that I have made the objects of careful microscopic examination.

In one place of the venous wall the elastic threads had been burst and disjoined by the invading yeast cells, which over a certain area entirely filled the vessel, while they, on other points, were covered by a thrombus. An examination of the inner organs of the animal showed that the yeast cells had already reached the liver and lungs, lying sparely spread inside all over the vessels, particularly numerous in the lungs.

In the groin there were three swollen lymph nodules. Two of them, of the size of a pea, contained a great many yeast cells. The third was about the size of a hempseed showing only a few parasites.

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The results of these investigations may be resumed as follows:

After subcutaneous inoculation of Saccharomyces neoformans Sanf. in guinea-pigs a lively emigration of polynuclear colourless corpuscles begins after a few hours. These corpuscles gather round the yeast cells that are lying in a heap, and ingest them. As soon as the greater part of the yeast fungi has been taken up by the cells, the emigration stops having entirely ceased eight hours after inoculation.

Besides the non-granular, polynuclear, white corpuscles a few are seen with acidophil granules. Their nucleus is, as a rule, two-lobed. They take a small part only in the phagocytosis, disappearing very soon.

After the lapse of 18—24 hours a number of mononuclear, non-granular, wandering cells are seen in the adjoining connective tissue without any connection with blood-vessels. These wandering cells seem due to a direct division of the connective tissue cells. They are penetrating into the yeast heap among the polynuclear phagocytes; like these they ingest yeast cells and, in a few places, they seem to fuse into giant cells [s. Fig 3]. The latter appear already after 3—4 days.

Gradually fibroblasts make their appearance, due, as it seems, to transformation of mononuclear wandering cells. The fibroblasts will either form a ring round the yeast cell heap or divide the same in smaller groups. Some of the yeast cells now alter their appearance. They increase in size, their staining alters, many of them get capsules; from „culture yeast cells“ they become „tissue yeast cells“. These altered fungi will always lie free and isolated, spread in the tissue. Later on, they increase greatly in number forming little heaps which are lying in a tissue of fibroblasts in the characteristic way described above: They make the impression as if they were lying in hollows which, in life, must be supposed to have been filled with a fluid. At the same time the fibroblasts multiply by indirect division, and especially on the fifth day a great many karyokinetic figures were seen [s. Fig 4].

Moreover, fine, new connective tissue threads are now perceptible. Whilst the injected *„culture yeast cells“* produced a violent acute inflammation with an active emigration of leucocytes, no anatomical changes of the kind are found later on during the quick growth of tissue yeast cells.

On the whole *„the tissue yeast cells“* seem to be acting chiefly in a *mechanical* way on the surrounding tissues. As soon as the greater part of the culture yeast cells have been ingested by the phagocytes the emigration stops, and most of the supervenient changes may well be explained as due to nothing but the increase in number of the yeast fungi. It is, however, natural to suppose that they affect the surrounding tissues in a chemical way as well, even though this occurrence was not very evident in the method of experimenting used here. The characteristic vacuolisation of mononuclear cells as described above, may perhaps be looked upon as the result of a poisonous action.

Rather early — on the fifth day — yeast cells may be shown in the lymph nodules next to the point of inoculation. Later on, their number increases greatly, and they propagate from one lymph nodule to the nearest one. Some swelling of these lymphatic glands is, however, always seen *before* the yeast cells can be pointed out. It is impossible from what has been said above to decide whether this fact is due to a toxic

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yeast cell product or to decaying matter from the tissues.

I beg to thank most heartily Professor Carl Weigert, under whose direction I have undertaken this work at the Senckenberg Institute of Frankfurt a/M., for his great kindness and his excellent guidance.

DESCRIPTION OF PLATE

All figures were drawn with Zeiss's camera lucida and apochromatic lenses.

Fig. 1. Guinea-pig 1. Lithium carmine + Weigerts elastic tissue stain $^{280}/_{1}$. From the border of the yeast cell group. a free yeast cells in the middle of the group, b immigration of leucocytes in the border, beginning phagocytosis, c emigration from a dermal vein.

Fig. 2. Guinea-pig 9. Lithium carmine + Claudius $^{280}/_{1}$. Tissue yeast cells lying in hollow spaces (*) among fibroblasts.

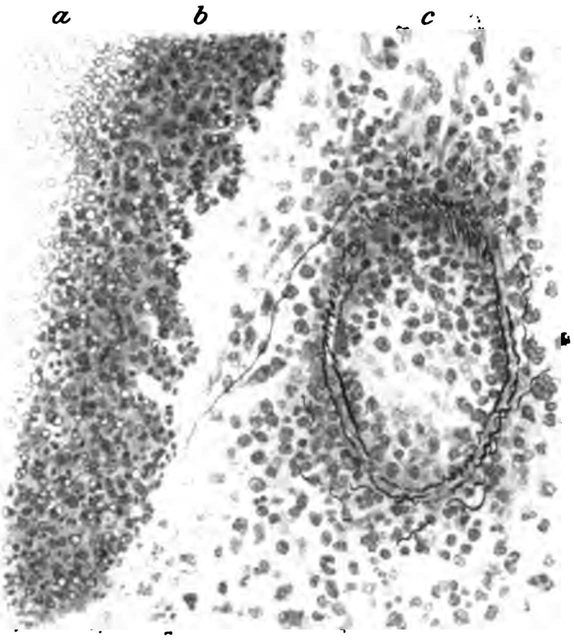
Fig. 3. Guinea-pig 5. Lithium carmine + Claudius $^{650}/_{1}$. Giant cell formation by mononuclear wandering cells.

Fig. 4. Guinea-pig 9. Lithium carmine $^{650}/_{1}$. Karyokinesis in fibroblasts.

Fig. 5. Guinea-pig 1. Lithium + Weigerts fibrin stain $^{1000}/_{1}$. Culture yeast cells, alterated in staining capacity due to their being for $3^{1}/_{2}$ hours in the tissue of the guinea-pig.

Fig. 6. Guinea-pig 6. Claudius $^{1000}/_{1}$. Tissue yeast cells.

Fig. 7. Guinea-pig 12. Hæmatoxyline + van Gieson $^{1000}/_{1}$. Vacuolated cells. Degenerated yeast cells in the vacuoles of one, granules in another, absence of formed elements in the vacuoles of the remaining two.



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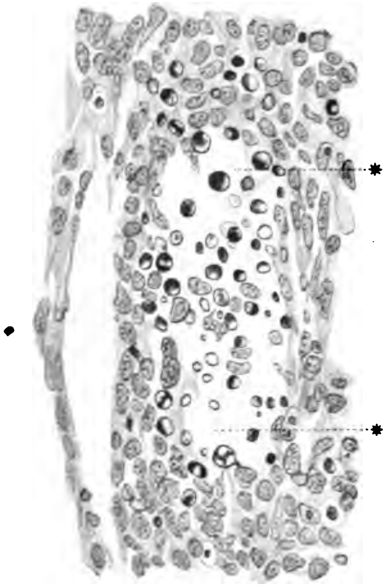

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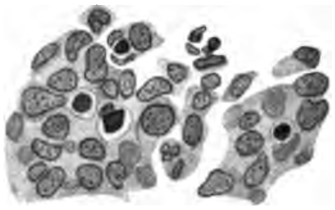

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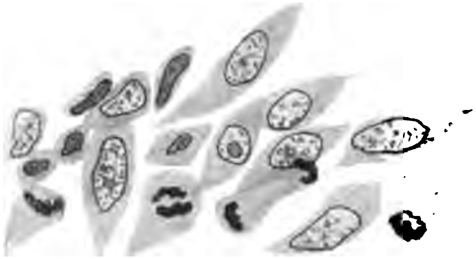

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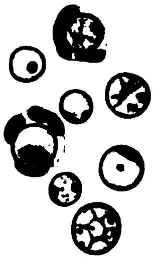

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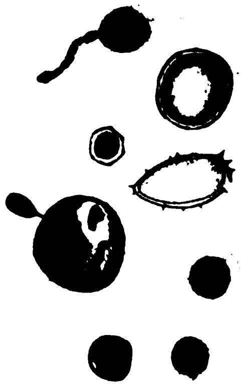

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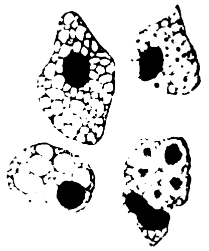

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XI.

GEORGES DREYER

A NEW APPARATUS FOR RAPID DELIVERY OF A GIVEN QUANTITY OF FLUID.

By the use of the following apparatus, devised for the rapid delivery of a *given quantity* of fluid, *sterile* or not, accurate results are obtained, and both time and labour saved, one person only being required to work it. Absolute exactitude and rapidity of flow are not only of importance when bottling anti-sera, but are equally important in measuring definite quantities of blood, cultures etc. for hæmolytic and agglutinin experiments respectively.

The apparatus consists in the main of two parts, the measuring apparatus itself, and the stand to which this is affixed.

The measuring apparatus as seen in front view (Fig. A) consists of a central branched tube of brass, a four way stopcock(e), and two graduated pipettes (c) connected by means of india rubber tubes with the side branches of the central tube. The pipettes used, are ordinary glass pipettes, having a capacity of 5—20 Cc. The upper ends are bent at a sharp angle and have only a small bore. Round this portion of each pipette a glass bulb is melted on, with an overflow tube (l) proceeding from it. The pipettes are attached by means of a moveable screw clamp (d) to the central tube.

The movement of the stopcock is brought about by the action of the pedals. The bore of the stopcock is so arranged that fluid cannot flow in either direction

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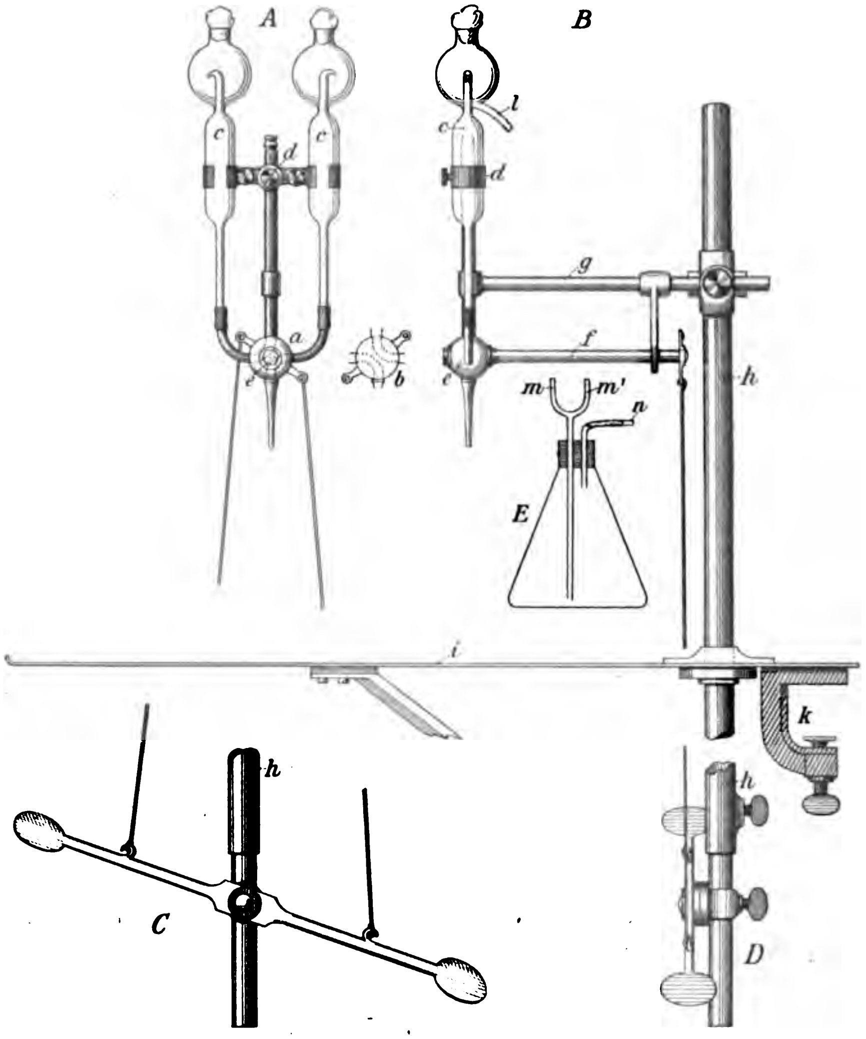
when the beam to which the pedal rods are hooked is in a horizontal position. When the stopcock is in position (a), fluid can pass from the central tube (attached



when in use to the reservoir of fluid) by the side branch up into the left pipette or into the right pipette if the stopcock is in position (b).

The measuring apparatus is attached by a support (g) and moveable clamp to the iron stand (h). This arrangement makes it possible to alter the distance between the outflow tube of the stopcock and the white enamelled metal plate (i). The latter forms the table of the apparatus and is supported by a metal bar fixed to the stand (h).

The stand can be fastened to any table by means of the two screw clamps (k) and the rigidity of the whole is ensured by its passing through the enamelled plate (i) to the floor.

To allow for the apparatus being fixed to tables of different height, the lower portion of the stand and the brass rods from the pedals to the stopcock are so made that they can be shortened or lengthened accordingly.

The form of pipette proposed by Kern¹) might be preferred by some to that mentioned above. Kern's pipette (provided it is carefully made) has this advantage, that there is no overflow or possible waste of fluid, but on the other hand the quantity of fluid delivered is not always the same, air bubbles of different size frequently collect under the float closing the pipette, and if a viscid fluid (serum, blood etc.) is used, the float will very often adhere to the side of the pipette and prevent this being emptied.

For these reasons, the above named pipette has been designed; owing to the small bore of the upper portion, the waste of fluid will be practically nil, and the formation of air bubbles prevented by the sharp angle bend.

If the apparatus is to be used for sterile fluid, it is arranged for sterilisation in the following way: an india rubber tube of required length and of such a width as

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¹) **Kern, F. „Eine automatische Messpipette für keimfreie Flüssigkeiten" Centralblatt für Bakteriol u. Parasitk. 1899. Bd XXV. p. 75.**

to be easily applied to the reservoir of fluid, is placed upon the central tube, the unattached end and the outflow tube of the stopcock being wrapped in paper; cotton wool corks are placed in the neck of the pipette-bulbs; the overflow tubes from the pipettes are connected by india rubber tubes with the branches (m—m[1]) of the tube in the Erlenmeyer flask (E) and the bent tube (n) in this, is corked with cotton wool.

By unscrewing the clamp attaching the pipettes to the stand, and unhooking the pedal rods from the beam of the stopcock, the measuring apparatus thus arranged with the Erlenmeyer flask, can be placed in the autoclave.

After sterilisation the apparatus is again fixed to the stand, the pedals hooked on and the rubber tube attached to the reservoir of fluid.

By the pedal action it will be noticed that as one pipette fills, the other is emptied, though it is so arranged that the filling process shall not be completed until after the contents of the other pipette have been entirely discharged, and, that when the beam of the stopcock is in a horizontal position there can be neither inflow nor outflow of fluid in any direction.

Further comment on the use of the apparatus for nonsterile fluids is unnecessary.

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1) **The apparatus can be obtained from: Cornelius Knudsen's mekaniske Etablissement, Copenhagen.**

Explanation of Figures.

A. Front view of measuring apparatus.
B. Side view „ „ „ and stand.
C. Front view of pedals.
D. Side view „ „
E. Erlenmeyer flask for overflow.
(a), (b) the two positions of stopcock.
(c) graduated pipettes.
(d) screw clamp (double).
(e) four way stopcock.
(f) axis on which pedals act.
(g) support attaching measuring apparatus to stand.
(h) metal stand.
(i) white enamelled metal plate.
(k) strong clamp.
(l) overflow tube.
(m,m[1]) branched tube.
(n) bent tube.

XII.

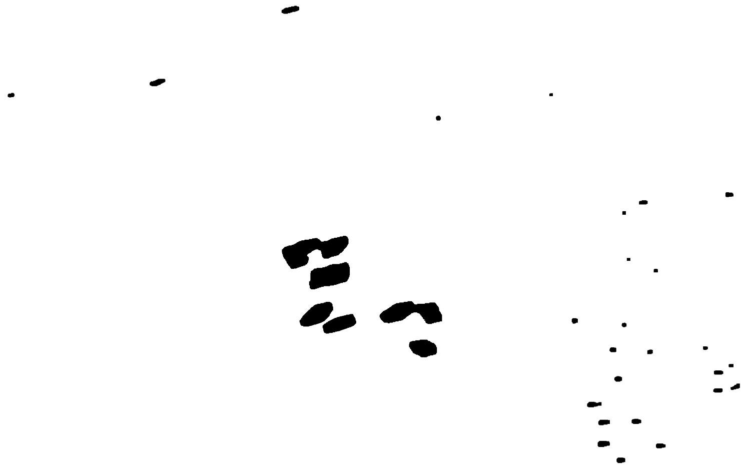
CARL JUL. SALOMONSEN

A CHEMOTACTIC EXPERIMENT

Cohnheim's experiments on inflammation and Metschnikoff's phagocyte theory have made investigations into the chemotaxis of monocellular organisms of the greatest importance to general pathology. From this point of view the following communication may perhaps be of some interest.

A phenomenon which till now has escaped observation is the negative chemotactic action that dead infusoria have on living ones. I observed this fact for the first time when, on repeating Mendelsohn's experiments on thermotropism, I happened to kill a few Oxytrichae by overheating.

a. The readiest way of making the experiment is by taking a drop containing plenty of infusoria of the same species and placing it under a lamella. If its surface then is touched for a moment with a platina needle or a glass-rod intensely heated, a more or less considerable number of infusoria will be killed. The live individuals will stop as soon as they approach the dead infusoria, often even at a very great distance, and withdraw so as to leave a circular space into which no living organism penetrates. A very vivid illustration of the occurrence may be had by drawing the dead bodies by means of a camera lucida and by following afterwards the movements of the living individuals exactly marking, at

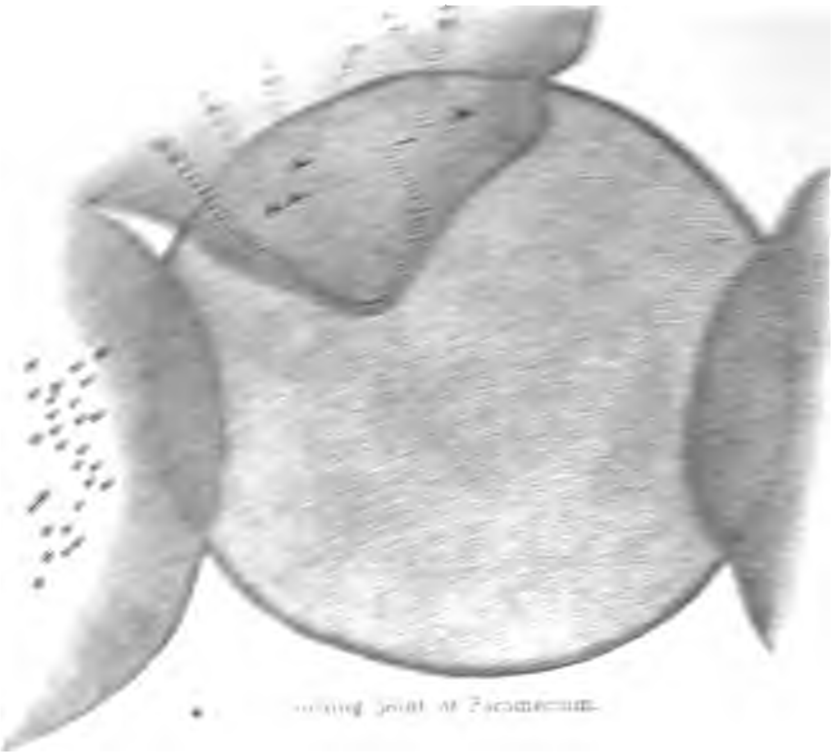


[illegible]

[illegible] means of the camera insu- [illegible] the animalcula are [illegible] made in this way [illegible] same for all the [illegible] depends on the pecu- [illegible] of each species. There [illegible] a more- [illegible] as Engelmann [illegible] in a curve, others again [illegible] before moving back. — A certain number react in a particular way at a somewhat greater distance from the dead bodies, executing a series of rapid rotations round their axes like the rotations which Rossbach observed during his experiments on the influence of some toxic substances on infusoria. It is well known that in preparations infusoria will often be seen rotating in this manner without any visible motive. According to the above observation I have no doubt that these rotations always are a *symptom of indisposition?* This interpretation is also confirmed by the fact that such rotations sometimes appear in the agonies of death, thus I have seen infusoria turn rapidly round their axes as often as 70 times immediately before dying.

c. The cause of death seems to be immaterial to this chemotactic action. It is found in dead bodies of infusoria whether killed by faintly heating, by ebullition, by exsiccation, by crushing, or by poison. In the latter case it is difficult to ascertain the fact on account of the complications resulting from the presence of the poison.

d. A question that presents itself is this: Is this reaction *specific?* In other words: Is it only on living infusoria of *their own species* that dead bodies have this effect? It is not unlikely a priori that this is so considering what is seen to take place in the processes of fecundation and of conjugation. — My very first experiments on this question seemed to favour the theory of specificity, but by further investigation I saw that there is no such exclusive reaction of living infusoria in the presence of dead ones of their own species. But, on the other hand, there is *a considerable quantitative difference* as to the force with which the different species of infusoria react against dead bodies of one and the same species.



• . . Turning point of Paramecium.

● . . Turning point of Paramecium.

▲ . . Turning point of Oxytricha.

.... Course of Vorticella.

The above figure shows both the arrangement of the experiment and an example of these differences. The circle in the middle represents a dried up drop on the slide, containing plenty of dead Paramecia. Next to this drop are placed those other drops which contain the different species of infusoria whose irritability in face of the dead bodies is to be examined. They must be placed so as to cover a small part of the dried drop. The black points mark the place where the *Oxytrichae turn.* The triangles have the same signification with regard to the *Paramecia;* whereas the asterisks mark the *whirling* points of the latter. The dotted line represents the course of a *Vorticella.* It is easy to see that the *Paramecia* draw back even before they reach the territory of the dead bodies; the *Oxytrichae* just penetrate into it, both turning round at a certain distance from the edge of the water drop in which they swim, whereas the *Vorticella* advances to the edge and even shirts it, without being influenced by the presence of the dead bodies.

c. *This insensibility seems to be peculiar to Vorticellae.* I have made analogous experiments on a great number of Ciliata of different species: they have all shown the same necrophobia except two species of Vorticella which remained totally insensible to dead bodies of foreign species, as well as of their own, and this to such a degree as to even touch the dead bodies in passing. The „sessility“ of the Vorticellae may have something to do with this exceptional quality, but at any rate we find this quality in the Vorticellae whether they be fixed or free swimming in the form of a campanula on its pedoncule or after having taken the form of a barrel.

It is still impossible for me to give any exact information concerning the substance or the substances having this strong chemotactic action.

The fact in question is perhaps not of great importance to general biology, but, no doubt, it is of interest as it permits us, by means of an easy, simple and, at the same time, most instructive experiment to show

(1) a typical case of negative chemotaxis,

(2) the great quantitative and qualitative differences

[illegible]
[illegible] Mcz,

between the chemotactic irritability of different species of Ciliata.

(3) The above method of experimenting may easily be applied to investigations into the influence of different external factors upon the chemotactic relations of monocellular organisms.

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